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MENGO VIRUS ASSEMBLY: *IN VITRO* SYNTHESIS, PURIFICATION AND CHARACTERIZATION OF THE 14S CAPSID PRECURSOR STRUCTURE

by

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To the glory of God

ABSTRACT

Mengo virus RNA was translated in a rabbit reticulocyte cell-free system to produce viral proteins. Sucrose gradient analysis of radioactively labelled translation products following a 16 hr incubation period revealed the formation of 5S [$\epsilon\alpha\gamma$] and 14S ([$\epsilon\alpha\gamma$]$_s$) particles, similar to those isolated from Mengo virus infected L-cells.

The 14S particles produced in both infected cells and reticulocyte lysates were purified by immunoaffinity chromatography on a column containing monoclonal antibodies specific for the capsid protein β. Mengo virus 13.4S subunits were used as eluant. These purified particles sedimented at 14S during recentrifugation and contained essentially pure proteins ϵ, α and γ, as judged from autoradiography and silver staining of SDS-PAGE gels. Preliminary assembly studies *in vitro* demonstrated that, in the presence of viral RNA, 14S particles from both sources are able to form structures having sedimention coefficients greater than 100S. However, virus-sized particles (150S) were only detected in mixtures of viral RNA and 14S particles purified from infected L-cells.

Two conclusions were drawn from studies in which 14S particles synthesized in the cell-free system and those isolated from infected L-cells were compared. (1) A morphopoietic factor responsible for facilitating the formation of 14S particles might be present in infected L-cells but not in the reticulocyte lysate since the

formation of 14S particles in the latter system was found to be 4 times slower. (2) The 14S particles produced *in vitro* might be conformationally different from those isolated from infected cells, since the two types of 14S particles exhibit distinctive behaviourial characteristics during the immunoaffinity chromatographic purification and in their interactions with viral RNA.

The amino terminus of protein ϵ was also examined. A chaotropic agent, lithium diiodosalicyte (LIS), was used to elute the 14S particles, ($[\epsilon\alpha\gamma]_s$), which had been specifically bound to the immunoaffinity column, as purified 5S particles, $[\epsilon\alpha\gamma]$. Automated sequence determination of ϵ, α and γ from this material was attempted, but no meaningful results were obtained, presumably because of the very limited solubility of these proteins after freeze-drying. Attempts to synthesize [^{3}H]acetyl CoA-labelled Mengo precursor proteins in the *in vitro* system also failed. Rapid conversion of [^{3}H]acetyl CoA into various amino acids resulted in the radioactive labelling of all newly synthesized viral proteins. It was found that both the initiation of synthesis and the post-translational processing of Mengo viral proteins in acetylation-inhibited reticulocyte lysates were markedly inhibited, and only small amounts of the precursor proteins were detected. Thus, the net result of these experimental approaches is that the amino terminal of polypeptide ϵ remains uncharacterized.

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LIST OF ABBREVIATIONS

BME	Eagle's basal medium
BMV	Brome mosaic virus
BSA	Bovine serum albumin
Ci	Curie
CpMV	Cowpea mosaic virus
B-RNA	Bottom component RNA
M-RNA	Middle component RNA
CS	Citrate synthase
DABITC	4-N,N-dimethylazobenzene-4'-isothiocyanate
DOC	Deoxycholate
DTT	Dithiothreitol
EDTA	Ethylene-diaminetetraacetic acid
EMC	Encephalomyocarditis
FCS	Fetal calf serum
FMDV	Foot and mouth disease virus
g	centrifugal force relative to gravity
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HPLC	High performance liquid chromatography
hr	hour
l	litre
LIS	Lithium diiodosalicylate
M	Molar
min	minute
mg	milligram

ml	millilitre
mM	millimolar
mmol	millimole
MW	Molecular weight
nm	nanometer
NP40	Nonidet P-40
OAA	Oxaloacetate
PBS	Phosphate buffered saline
pfu	plaque forming units
p.i.	post infection
RNA	Ribonucleic acid
rpm	revolutions per minute
RSB	Reticulocyte standard buffer
S	Sedimentation coefficient (svedbergs)
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TCA	Trichloroacetic acid
TLC	Thin layer chromatography
Tris	Tris(hydroxymethyl)aminomethane
µg	microgram
µl	microlitre
µM	micromolar

All temperatures are in degrees Celsius.

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1. INTRODUCTION

Mengo virus was first isolated from monkeys, mosquitoes, a mongoose and a laboratory worker at a monkey colony in the Mengo District of Buganda, Uganda. It was shown to produce lesions in the brains and spinal cords of infected animals and encephalitic symptoms in man (Dick, 1948). The virus was subsequently adapted to propagate in laboratory mice and in cultured mouse cells (Ellem and Colter, 1961). Mengo virus has been classified as a member of the family Picornaviridae (Matthews, 1982). The Picornaviridae comprise four genera of small mammalian RNA viruses, and Table 1.1 lists some of the physical properties of the species in each genus (Rueckert, 1976; Scraba, 1979; Putnak and Phillips, 1981).

Chemical analysis of picornaviruses has shown that 70% of the weight of the virion is accounted for by capsid proteins and 30% is RNA; neither lipids nor carbohydrates have been found. Sodium dodecylsulfate-polyacrylamide gel electrophoretic (SDS-PAGE) analysis of picornavirions has revealed four major capsid proteins, which are present in roughly equimolar ratios (Maizel *et al.*, 1967; Summers and Maizel, 1968). These have been designated VP1, VP2, VP3 and VP4 in the case of polioviruses, rhinoviruses and aphthoviruses, and α, β, γ and δ in the case of the cardioviruses. To simplify the situation, in this thesis the cardiovirus nomenclature will be used for all picornaviruses. This convention also implies that the

Table 1.1 Classification and physical properties of picornaviruses.

Genera	Type species or examples	Acid stability (in presence of Cl^-)	Buoyant density in CsCl (g/cm^3)	Sedimentation coefficient (svedbergs)	Empty capsids produced *in vivo*
Enteroviruses	Polioviruses Coxsackie viruses Echoviruses Animal enteroviruses	yes	1.33 -1.34	155	yes
Cardioviruses	Encephalomyocarditis virus Mengo virus Maus-Elberfeld virus	no	1.33 -1.34	155	no
Rhinoviruses	Human rhinoviruses	no	1.38 -1.42	155	yes
Aphthoviruses	Foot and mouth disease virus	no	1.43 -1.45	145	yes

corresponding proteins are similar in terms of size, gene order and structural roles; this certainly seems to be true based upon the data available (Putnak and Phillips, 1981). One to two copies of the capsid protein precursors ϵ (β + δ) and D2 (α + γ) are also found in each Mengo and EMC virus particle, though their function is not yet clear (Butterworth and Rueckert, 1972b; Ziola and Scraba, 1974).

The structure of the polio virion was first proposed by Finch and Klug (1959) to be a regular icosahedron, based upon the X-ray diffraction pattern obtained from crystals of the virus. Since there could be more than one possible arrangement for equimolar amounts of four capsid proteins in an icosahedral shell, the architecture of picornaviruses was, for a time, mysterious. Dunker and Rueckert (1971) made use of the acid lability of the cardioviruses to provide an insight to the structure problem. In the presence of chloride ions at slightly acidic pH, cardioviruses dissociate into 13-14S subunits which contain equimolar amounts of the capsid proteins α, β and γ. The fourth capsid protein, δ, is found in a precipitate together with the RNA. The 13-14S subunit could be further dissociated by 2 M urea into particles which sediment at about 5S. A molar ratio of 1:1:1 for the proteins α, β and γ is still maintained in the 5S particles. From the protein compositions and the molecular weights of the proteins and the particles, the 5S particle or protomer was found to contain only one copy of each of the three capsid proteins α, β and γ, while the

13-14S subunit was a pentamer ($[\alpha\beta\gamma]_5$). The 5S protomers probably associate by hydrophobic interactions to form a pentamer, and the pentamers associate by acid-sensitive electrostatic interactions to form the virus capsid. The dissociation scheme for Mengo virus is shown in Fig. 1.1.

Each picornavirus particle contains one molecule of infectious, single stranded RNA, with a molecular weight about 2.6×10^6 (Lee *et al.*, 1979). It is of the positive sense since it is translated directly during infection and can also be translated in cell-free systems (Smith, 1973; Villa-Komaroff *et al.*, 1974; Pelham, 1978). It shares with most eukaryotic messenger RNA's a 3'-poly(A). However, this poly(A) segment is not added post-transcriptionally by enzymes; it is transcribed from the poly(U) template at the 5'-end of the negative strand RNA during replication. The presence of poly(A) is essential for the infectivity of the RNA, but it is not clear whether there is minimum requirement for the length of the poly(A) chain (Putnak and Phillips, 1981). A poly(C) tract about 100 residues long is found near the 5'-end of the RNA of cardioviruses (and aphthoviruses; Fellner, 1979). No function has been ascribed to the poly(C) tract.

Unlike most eukaryotic messenger RNA's, picornavirus RNA does not contain a 5'-cap structure. A small protein designated VPg (Lee *et al.*, 1977; Sanger *et al.*, 1977; Hruby *et al.*, 1978) is covalently linked to the 5'-terminus of the viral genome through an O⁴-(5'-uridylyl)tyrosine

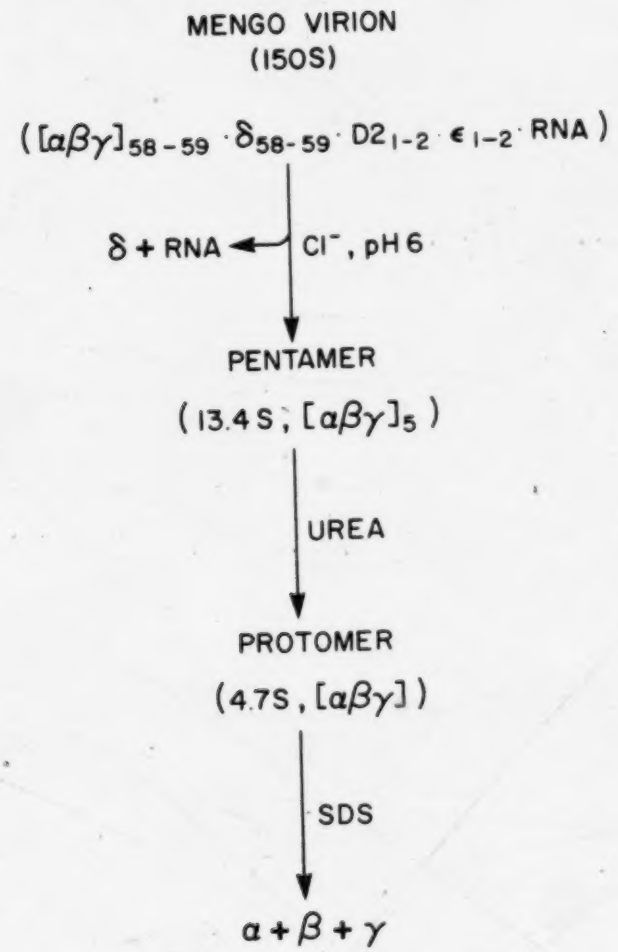

Fig. 1.1 Dissociation of the Mengo virion.
In the presence of 0.1 M Cl^- at pH 6, the Mengo capsid dissociates into 'soluble' 13.4S pentameric subunits. The δ polypeptides along with the viral RNA (and the 'immature' protomers containing ε and/or D2 polypeptides) form a precipitate. The 13.4S subunits can be further dissociated into 4.7S protomers (containing one molecule each of polypeptides α, β, and γ) by treatment with 2 M urea. The individual polypeptides can be released from the protomers by heating in the presence of 1% SDS. (From Mak *et al.*, 1974).

linkage. This virus-encoded protein is not required for translation but is involved in the replication of viral RNA (Wimmer, 1979). Translation and replication of picornaviral RNA takes place in the cellular cytoplasm. VPg is removed from the RNA molecule by a cellular enzyme before translation begins (Nomoto *et al.*, 1977; Ambros *et al.*, 1978). Once the translation of the viral mRNA has started, synthesis of host cell proteins is inhibited markedly (Franklin and Baltimore, 1962; Lucas-Lenard, 1979). This 'shut-off' of host cell protein synthesis is common for all picornaviruses, and greatly facilitates studies of the synthesis and processing of viral proteins. The infected cell lysates can be analyzed by SDS-PAGE and the newly synthesized viral proteins visualized by autoradiography with little interference from host proteins (Summers *et al.*, 1965).

It is generally accepted that picornavirus RNA is monocistronic (Jacobson and Baltimore, 1968a) and has a single initiation site for translation. Based on these assumptions, the gene order and precursor-product relationship of various viral proteins synthesized in infected cells and cell-free translation systems have been determined by pulse-chase experiments, pactamycin mapping and tryptic peptide identifications (Butterworth and Rueckert, 1972a, 1972b; Paucha *et al.*, 1974; Shih *et al.*, 1978; Grubman, 1984). Shown in Fig. 1.2 is a processing map of Mengo viral proteins. This is a modification of the

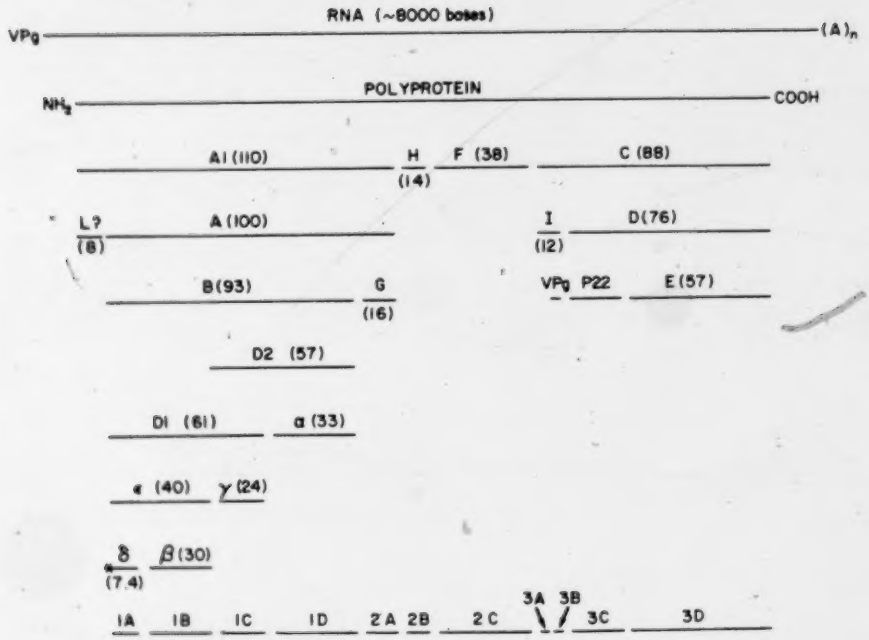

Fig. 1.2 Gene order and cleavage map of Mengo viral proteins.
Mengo viral RNA is translated into a polyprotein of MW ~250,000 both *in vivo* and *in vitro*. The polyprotein is unstable, being cleaved while nascent by a cellular enzyme(s) into three segments: A1, H & F, and C (Korant *et al.*, 1980). The structural precursor A1 can be detected in the *in vitro* translation system, but (by analogy with EMC virus; Palmenberg *et al.*, 1984) *in vivo* a leader peptide (L) is immediately removed from the amino terminal portion of A1 to generate A. The other secondary cleavages shown were determined by pulse-chase experiments, and the gene order of the structural and nonstructural proteins by pactamycin mapping experiments (Paucha *et al.*, 1974). These cleavages are mediated by a viral protease, most likely p22 (Rueckert *et al.*, 1980). The 'morphogenetic' cleavage of ϵ to δ + β occurs during the final steps of progeny virion formation. The δ polypeptides isolated from virions have blocked amino-termini (Ziola and Scraba, 1976); neither the nature of the blocking group nor the stage in post-translational processing where blocking occurs have been determined. Polypeptide C, representing the 3' part of the RNA coding region, is the precursor for the 5' genome-linked protein, VPg, the protease, p22, and the polymerase, E (Lund and Scraba, 1979). The molecular weights given are those determined from SDS-PAGE. The standard 4-3-4 nomenclature for picornavirus polypeptides (Rueckert and Wimmer, 1984) is included at the bottom of the figure.

scheme proposed by Paucha *et al.* (1974) using the nucleotide sequence data determined for encephalomyocarditis (EMC) virus (Palmenberg *et al.*, 1984). Translation of picornaviral RNA starts very close to the 5'-end of the molecule and proceeds along the whole genome to produce a polyprotein with molecular weight greater than 220,000. This polyprotein is not stable and is cleaved by host cell enzymes to the protein precursors A1, F and C before its synthesis is completed. Only in the presence of amino acid analogs (Jacobson and Baltimore, 1968a; Jacobson *et al.*, 1970; Paucha *et al.*, 1974), zinc ions (Butterworth and Korant, 1974), or protease inhibitors (Korant, 1972; Summers *et al.*, 1972) can the polyprotein be detected. As shown in Fig. 1.2, the viral capsid and noncapsid precursors are further processed into their final products by the action of a protease which is believed to be of viral origin. The two possible candidates are proteins F (Korant *et al.*, 1979) and p22 (Palmenberg *et al.*, 1979). P22 autocatalytically removes itself from precursor D (Rueckert *et al.*, 1980); the resulting polypeptide E, which is also a stable viral protein product, is the RNA replicase of the virus (Traub *et al.*, 1976; Flanegan and Baltimore, 1977; Lund and Scraba, 1979).

The first cardiovirus capsid protein precursor shown in Fig. 1.2 is protein A1. This precursor was not detected in infected cells unless the primary processing of precursor proteins was inhibited by zinc ions or amino acid analogs

(Paucha *et al.*, 1974; Lucas-Lenard, 1974; Butterworth and Korant, 1974). The corresponding EMC viral protein synthesized in a rabbit reticulocyte lysate (originally termed 'pre-A') was shown by Pelham to be the precursor of protein A (Pelham, 1978). A leader peptide (L) produced by the cleavage of A1 has been identified for EMC (Campbell and Jackson, 1983) but not for poliovirus. In the latter case, the amino terminal of the capsid precursors is conserved during subsequent processing (Dorner *et al.*, 1982). Processing of protein A gives rise to another precursor, B. Cleavage of B follows two routes. It is either cleaved into D1 and α, or is cleaved into ε and D2. The latter pathway is thought to be followed in Mengo infected cells, since D2 is detected in Mengo virus particles (Ziola and Scraba, 1974).

The amino terminus of the capsid protein δ of all picornaviruses is blocked. The nature of the blocking group is not known. Functionally, blocking of the amino terminal may protect the protein from exopeptidase degradation, and/or the neutralization of 60 positive charges brought about by the blocking groups may play an important role in the assembly of the virion. It has been shown that all of the precursor proteins containing the amino terminus of the poliovirus polyprotein are blocked (Dorner *et al.*, 1982). Whether the amino termini of capsid precursors (A, B, D1 and ε) of cardioviruses are also blocked is not known. However, a difference may be anticipated because of the presence of the peptide L in the precursor of the cardioviral capsid

proteins.

The final cleavage of ϵ to β and δ has not been detected in cell-free translation systems programmed with picornavirus RNA; neither are mature virions produced in these systems (Shih *et al.*, 1978). Empty capsids isolated from poliovirus infected cells contain only proteins ϵ, α and γ (Maizel *et al.*, 1967). These data support the proposal of Jacobson and Baltimore (1968b) that the cleavage of ϵ occurs during the incorporation of the viral RNA into a pre-formed capsid.

Assembly of virus particles follows the synthesis of viral structural and nonstructural proteins and the production of progeny RNA molecules. Although many important details of the assembly process are not clear, a model shown in Fig. 1.3 has been proposed based on the available information (Scraba, 1979; Putnak and Phillips, 1981). Readers should refer to this model when various pathways and intermediates are being described. A series of subviral particles, at least some of which are assembly intermediates, have been isolated by ultracentrifugation. These include particles sedimenting at 5-6S, 13S and 14S (Rueckert *et al.*, 1969; McGregor *et al.*, 1975). For poliovirus, 80S 'procapsids' ($[\epsilon\alpha\gamma]_{60}$; Jacobson and Baltimore, 1968b) and 125S 'provirions' ($[\epsilon\alpha\gamma]_{60}$ RNA; Fernardez-Tomas and Baltimore, 1973) have also been described. Empty capsids have not been detected in cardiovirus infected cells, but a 53S particle ($[\epsilon\alpha\gamma]_{25}$) was

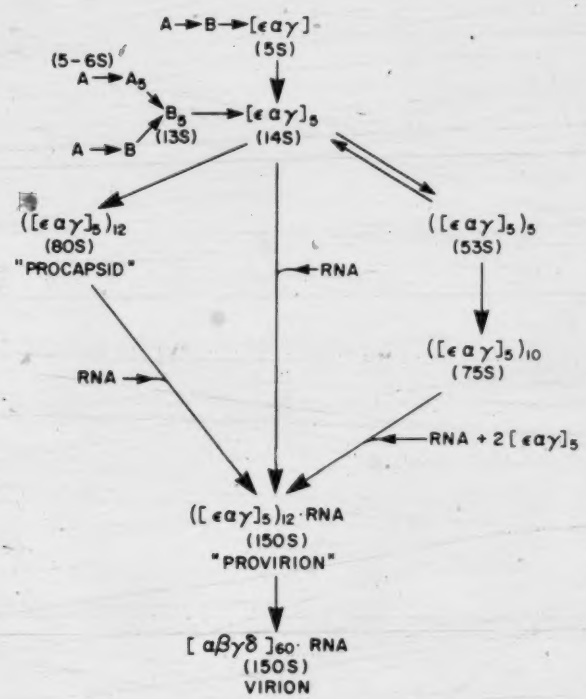

Fig. 1.3 Model for picornavirus assembly showing the structural intermediates and hypothetical pathways.
In infected cells capsid precursor molecules (either A or B) associate to form pentamers, and concomitant cleavage produces a 14S particle whose composition is $[\epsilon\alpha\gamma]_5$. In the *in vitro* protein synthesizing systems programmed with cardiovirus RNA, the precursor proteins are cleaved to produce $[\epsilon\alpha\gamma]$ protomers which are subsequently assembled into 14S particles. There are three possible pathways whereby mature virions are formed from the 14S pentamers. On the left is the pathway proposed for poliovirus (Jacobson and Baltimore, 1968b; Fernandez-Tomas and Baltimore, 1973) which involves the formation of a 'procapsid' followed by the incorporation of virion RNA and the final 'morphogenetic' cleavage of the ϵ polypeptides to β and δ. A direct interaction between the RNA and 12 of the 14S particles is also a possibility (center pathway), with condensation producing the 'provirion' (Ghendon *et al.*, 1972). Finally, on the right is shown the pathway proposed for Mengo virus by Lee and Colter (1979). This involves the formation of 53S and 75S intermediate structures (five and ten 14S particles?) followed by the incorporation of the RNA and addition of other 14S particles to complete the capsid. Although not shown here, the final maturation step leaves one or two copies of uncleaved ϵ in each picornavirion.

isolated from Mengo infected cells (Lee and Colter, 1979).

Particles which sedimented in the 5S region of sucrose density gradients were found to contain a single molecule of capsid protein precursor A. Also detected in the 5S region were particles containing ϵ, α and γ, the cleavage products of precursor A (McGregor *et al.*, 1975; McGregor and Rueckert, 1977; Shih *et al.*, 1978). Precursor A produced in EMC virus infected cells was also found in a structure composed of five A molecules, which sedimented at about 13S (McGregor *et al.*, 1975). A structure sedimenting at about 14S (the 14S particle) consisted of five molecules of each of ϵ, α and γ, and could be a pentameric association of the cleaved 5S particles or a processed form of the 13S particles ($[A]_5$).

The 14S particle is thought to be a legitimate assembly intermediate. This particle is rapidly labelled in infected cells, and poliovirus 14S particles have been shown to be able to assemble into empty capsids in the presence or absence of infected cell extracts (Putnak and Phillips, 1982). Since self-assembly of 14S particles and assembly of 14S particles in the presence of uninfected cell lysates do not give rise to a structure identical to the empty capsids found in infected cells ('procapsids'), but rather to one which has a different isoelectric point and sensitivity to proteases, the requirement for a morphopoietic factor encoded by the virus was postulated.

It is tempting to conclude that in the assembly of virions, 80S 'procapsids' are produced from 14S particles and the viral RNA is encapsidated by some means afterwards. However, the absence of stable empty capsids in cardiovirus infected cells suggests the possibility of alternate pathways. Progeny viral RNA may be encapsidated directly through interaction with the 14S particles. The 80S 'procapsids' or the 53S particles mentioned above may represent a storage form of the 14S particles.

To elucidate the details of picornavirus assembly, the postulated intermediates and factors must first be isolated and purified in order to study assembly reactions under defined conditions *in vitro*. The only kind of *in vitro* assembly reaction reported to date is the assembly of poliovirus 14S particles into procapsids, which was mentioned earlier. However, the 14S particles used in these studies were far from pure. Phillips and Wiemert (1978) examined the degree of contamination in poliovirus 14S particles prepared from sucrose gradients and estimated that 80% of the protein present in these preparations was of host origin. In addition, the viral proteins present in the 14S particles were not only ϵ, α and γ; detectable amounts of other structural precursors and nonstructural polypeptides were also present. In contrast, 14S particles which contained only the capsid proteins ϵ, α and γ (and probably some unlabelled cellular proteins) were isolated from an EMC virus RNA directed cell-free translation system derived from

a rabbit reticulocyte lysate (Palmemberg, 1982). With the removal of contaminating reticulocyte proteins, these 14S particles could serve as suitable intermediates for *in vitro* assembly studies.

One of the aims of the project described in this thesis was to synthesize 5S and 14S particles in a Mengo virus RNA directed cell-free translation system and to purify these particles so that they could be used to study the process of virus assembly under defined conditions.

The amino-terminus of capsid precursor protein ϵ was also investigated. The structural proteins ϵ, α and γ from purified 5S particles isolated from Mengo virus infected cells were subjected to automated N-terminal sequencing. Since the amino terminal sequences of capsid proteins α and γ are known (Ziola and Scraba, 1976), the appearance of a third amino acid in each sequencing cycle would have been contributed by ϵ. If this were found to be the case, then the Mengo capsid precursor protein ϵ would have a free amino terminus, and the N-terminal modification of δ must occur during the maturation of the virion. Palmiter (1977) depleted the acetyl CoA from a rabbit reticulocyte translation system by the addition of oxaloacetate and citrate synthase, and discovered that ovalbumin (which is normally acetylated at its amino terminus) could then be synthesized from this system in a form that was amenable to automated sequencing. Since acetylation of proteins is very common in eukaryotic systems, the possibility that ϵ is an

acetylated protein was examined with the aid of this acetylation-inhibited *in vitro* translation system.

2. MATERIALS AND METHODS

2.1 Virus Growth and Purification

The M plaque variant of Mengo virus used was originally isolated by Ellem and Colter (1961). Confluent monolayers of mouse L-929 fibroblast cells were grown in roller bottles (1250 cm^2, ~3×10^8 cells/bottle) and infected with virus at a multiplicity of 20 plaque-forming units (pfu)/cell. After 18 hr incubation at 37°, the medium (Eagle's basal medium [BME] containing 1% fetal calf serum [FCS]) and cells were collected and centrifuged at 1,000 *g* for 15 min. The supernatant was removed and the cell pellet was resuspended in water, frozen and thawed twice to release trapped virus, and recentrifuged. The two supernatants were combined and chilled and the virus was precipitated by adding cold methanol to a final concentration of 33%. The solution was stored at -20° for at least 6 hr and the virus precipitate was collected, homogenized, and digested with α-chymotrypsin (3x crystallized, Worthington Biochemicals, Freehold, N.J., USA) to remove cell debris. The virus was pelleted from the supernatant by centrifugation and was further purified by centrifugation through a sucrose gradient, equilibrium centrifugation in cesium sulfate and gel filtration in G25 Sephadex according to the procedure described by Ziola and Scraba (1974). The quantity and purity of virus preparations were determined from the optical absorption at 260 nm, the A_{260}/A_{280} ratio and the ultraviolet absorption spectrum

(Scraba *et al.*, 1967). The infectivities of virus preparations in terms of plaque-forming units (pfu) were determined by the plaque assay method, using monolayers of mouse L-cells, as described by Campbell and Colter (1965).

2.2 Preparation of Viral Ribonucleates

Cowpea mosaic virus (CpMV) middle and bottom components were generous gifts from Dr. J.E. Johnson (Purdue University). RNA's were isolated from these viral components as well as from purified Mengo virus by a procedure employing phenol extraction as described by Scraba *et al.* (1967). The resulting RNA solution was made 0.2 M in sodium acetate and the RNA was precipitated with an equal volume of absolute ethanol. Dried RNA pellets were redissolved in sterile distilled water to give a final concentration of 1-2 μg/μl, and were stored at -20° in 10 μl aliquots. The purity and integrity of the RNA was checked by analytical ultracentrifugation; intact Mengo RNA has an $S_{20,w}$ value of 35 (Scraba *et al.*, 1967).

Brome mosaic virus (BMV) mRNA was purchased from Promega Biotec (Madison, Wis., USA).

2.3 Translation of Viral RNA in the Rabbit Reticulocyte Lysate

Nuclease treated rabbit reticulocyte lysates (Promega Biotec) were stored in liquid nitrogen in 200 μl aliquots. Seventy percent of the volume of a standard protein

synthesis mixture (total volume 60 µl) was made up of the rabbit reticulocyte lysate. The system was also supplemented with 1.2 µl of 1 mM methionine free amino acid mixture (Promega Biotec) and about 1 µM of [^{35}S]methionine (about 1000 Ci/mmol, New England Nuclear, Boston, Massachusetts, USA). Five µg of Mengo viral RNA were added to the translation mixture, and incubation was at 30° for 16 hr unless stated otherwise. After incubation, bovine pancreatic ribonuclease A (Sigma Chemical Co., St. Louis, Mo., USA) was added to a final concentration of 33 µg/ml and the sample was incubated at 30° for a further 45 min. The translation mixture was then diluted with one volume of cold reticulocyte standard buffer (RSB; 10 mM NaCl, 10 mM Tris-HCl, 1.5 mM $MgCl_2$, pH 8.5) and loaded onto sucrose gradients for isolation of structural intermediates.

Translation of Mengo virus RNA, CpMV RNA's and BMV RNA's were also carried out in the presence of 1 mM oxaloacetate (Sigma, Grade I) and 30 units/ml citrate synthase (Sigma, crystalline suspension of 1923 units of enzyme/ml in 2.2 M ammonium sulfate) to deplete the translation system of acetyl CoA. Preparation of oxaloacetate (OAA) stock solutions and recovery of citrate synthase (CS) from the ammonium sulfate precipitate were done as described by Palmiter (1977). The amount of rabbit reticulocyte lysate was reduced to 55% of the total volume to allow for the addition of OAA and CS. The total volume of protein synthesis mixtures of this sort was usually 12 µl,

and contained 1 μM of [^{35}S]methionine (~1000 Ci/mmol, NEN). Appropriate amounts of OAA and CS were added just before the addition of RNA. Mengo viral RNA and BMV RNA's were added to a concentration of 1 μg per 12 μl (83 μg/ml), and CpMV M or B RNA's were added at a concentration of 0.72 μg per 12 μl (60 μg/ml) translation mixture, which also contained 2 mM dithiothreitol (DTT; BIO-RAD Laboratories, Richmond, Calif., USA). Incubation was at 30° for 5 hr unless stated otherwise. In some experiments 3 μM [^{3}H]-acetyl CoA (about 7 Ci/mmol; NEN) was used as the radioactive label.

2.4 Translation of Mengo Virus RNA in the Wheat Germ Cell-free System

The standard protein synthesis mixture (30 μl) contained 10 μl of nuclease treated wheat germ extract ('3-fold concentrated'; Bethesda Research Laboratories, Gaithersburg, MD, USA) plus the following components: 5.5 mM creatine phosphate, 0.2 mg/ml creatine kinase, 80 μM spermidine phosphate, 1.2 mM ATP, 0.1 mM GTP, 50 μM each of 19 amino acids (except methionine), 1.8 mM 2-mercaptoethanol, 97 mM potassium acetate, 2.4 mM magnesium acetate, 0.8 μM [^{35}S]methionine (about 1000 Ci/mmol, NEN), and 2 μg of Mengo virus RNA in sterile distilled water. In some cases 30 units of the human placental ribonuclease inhibitor RNasin (Promega Biotec) and 1 mM DTT were also included. Incubation was at 25° for 5 hr.

2.5 Preparation of Infected Cell Lysates

Purified Mengo virus was added to a confluent monolayer of L-cells in a roller bottle at a multiplicity of 100 pfu/cell. One hour later (zero time post infection), the medium was replaced by BME containing 4% FCS. After 3 hr, 250 µCi [^{3}H]leucine (157 Ci/mmol, NEN) or 1 mCi [^{35}S]methionine (~1000 Ci/mmol, NEN) in medium deficient in the appropriate amino acid was added to each bottle. At 4 hr p.i., the medium was removed and the cells were detached from the bottle by washing with physiological buffer containing EDTA and glucose. Cell lysates were prepared according to the method described by Marongiu *et al*. (1981). The cells were washed in PBS and lysed by Dounce homogenization in RSB (pH 8.5). Nuclei were removed by centrifugation and the supernatant was made 1% in NP40 and DOC in order to dissolve the cytoplasmic membranes.

2.6 Sucrose Density Gradient Centrifugation

Infected L-cell lysates (1 ml) or RNase digested standard 16 hr translation mixtures (diluted to 120 µl with 60 µl of RSB) were layered onto 11 ml linear 5-20% (w/v) sucrose gradients made in RSB (pH 8.5). Centrifugation was at 37,000 rpm for 15 hr (4°) in a Beckman SW41 rotor. At the end of the run, the gradients were fractionated into 270 µl fractions from the bottom of the gradients. The positions of radioactive proteins and subviral assemblies were detected either by the trichloroacetic acid (TCA) filter test (see

below) or direct liquid scintillation counting. Eleven ml linear 15-30% (w/v) sucrose gradients in either RSB (pH8.5) or 0.1 M sodium phosphate (pH 7.4) with or without 1% NP40 were also used for the centrifugal separation of viral particles larger than the 14S pentamers.

2.7 Trichloroacetic Acid Filter Tests

Incorporation of [^{35}S]methionine into proteins synthesized in cell-free translation systems was measured by adding 5 µl aliquots of translation mixture to 1 ml of 1 N NaOH, 2% H_2O_2 in 10 ml test tubes and incubating for 10 min at 37°. This served to destroy the TCA precipitable radioactive peptidyl t-RNA complexes and to oxidize the endogenous hemoglobin, thereby removing the red colour from the solution. Twenty µl of 0.1% bovine serum albumin (BSA) and 4 ml of cold 25% TCA were then added and the mixtures were incubated on ice for 30 min. The precipitates were collected on GF/C glass microfibre filters (2.4 cm, Whatman) in a Millipore Sampling Manifold. The test tubes were rinsed and the filters were washed with 10 ml cold 8% TCA, followed by 4 ml of acetone after which they were dried under vacuum. The dried filters were subjected to liquid scintillation counting.

Twenty µl samples from the sucrose gradient fractions were spotted onto GF/A glass microfibre filter disks (2.4 cm, Whatman). Twenty filters were placed in a filter holder and were then washed twice with ice cold 15% TCA and

once with ice cold 5% TCA. Finally, the filters were washed with ice cold acetone and dried under infrared lamps. Radioactivities were quantitated by liquid scintillation counting.

2.8 Gel Electrophoresis and Autoradiography

Phosphate-urea gels (10% acrylamide) were prepared according to the recipe of Palmenberg *et al.* (1979), except that 2.5 M urea was used instead of 5 M urea. Gradient or antibody column fractions to be analyzed by gel electrophoresis were combined and proteins precipitated with TCA in the presence of BSA carrier. The precipitates were washed twice with acetone. Twenty µl of two-fold concentrated sample buffer (2x sample buffer; 2.8% SDS, 0.8% 2-mercaptoethanol, 0.06% bromophenol blue, 20% glycerol in 0.01 M sodium phosphate buffer, pH 7.2) were added to the dried precipitate and the samples were placed in a boiling water bath for 5 min. Equal volumes of 5 M urea were added just before the samples were loaded onto the gel. Samples from the *in vitro* translation mixtures were diluted with five volumes of two-fold concentrated sample buffer without TCA-acetone precipitation. These samples were also heated and then diluted with 5 M urea as described above.

Electrophoresis in a BioRad slab gel electrophoresis apparatus (Model 221) was at 40 amp per gel for 22 hr. The gel was then removed, fixed in 40% methanol with 10% acetic acid, treated with EN³HANCE (NEN) and heat dried on Whatman

3MM filter paper in a BioRad slab gel dryer. The dried gel was then exposed to Kodak X-OMAT AR film (XAR-5) at -70°.

2.9 Preparation of Mengo Virus 13.4S Subunits

Purified Mengo virus ([^{3}H]leucine labelled, or unlabelled) was pelleted in a Beckman Type 70Ti rotor at 40,000 rpm for 60 min (4°). The virus pellet was resuspended in phosphate buffered saline (PBS), pH 6.2, at a final concentration of 1-1.5 mg virus/ml. Pancreatic ribonuclease A was added to a final concentration of 2 µg/ml and the virus solution was stirred at 37° for 1 hr. The suspension of dissociated virus was then loaded unto 11 ml 5-20% sucrose gradients in RSB (pH 8.5) and centrifuged at 35,000 rpm for 17 hr (4°) in an SW41 rotor. Gradient fractions containing 13.4S subunits were combined, dialyzed against RSB overnight and stored at 4°.

2.10 Coupling of Antibodies to Sepharose

Murine hybridoma cell lines producing monoclonal anti-Mengo antibodies were injected into mice, and IgG was subsequently purified from the ascites fluid by gel exclusion chromatography (Sepharose S200), and ammonium sulfate precipitation. Finally, the antibodies were dialyzed against 0.2 M sodium bicarbonate buffer (pH 8.0) containing 0.3 M NaCl. Monoclonal antibodies against the Mengo virus protein β thus purified were a gift from Mr. J. Bowen (Department of Biochemistry, University of Alberta).

Anti-BSA antibody was obtained from Miles Scientific (Naperville, Illinois, USA). Freeze dried CNBr-activated Sepharose 4B (Pharmacia, Dorval, Quebec) was washed with large volumes of 1 mM HCl and mixed overnight at 4° with the appropriate antibody in coupling buffer (0.25 M $NaHCO_3$, 0.5 M NaCl, pH 8.5). Unbound antibodies were then removed by washing, and the remaining active groups on the Sepharose were blocked by 1 M ethanolamine (pH 8.6). The antibody-Sepharose was then treated by three washing cycles, each cycle consisting of a wash at pH 8.5 (coupling buffer) followed by a wash at pH 4.0 (0.1 M CH_3COONa, 0.5 M NaCl). The end product was dried under vacuum, resuspended in an equal volume of RSB and stored at 4°.

2.11 Immuno-Affinity Chromatography

The antibody-Sepharose suspension (200 µl) was poured into a siliconized 1 ml Eppendorf pipette tip, the bottom of which was loosely plugged with glass wool. This immuno-affinity column was washed with RSB before the pooled samples containing 14S peaks from 6-10 sucrose gradients were slowly pumped through (loading). The effluent was recycled through the column once more, after which the column was washed with RSB containing 1% NP40, and then with RSB alone (washings). The bound 14S particles were eluted either by equilibration with 13.4S subunits prepared from dissociated virus, or by a chaotropic reagent, lithium diiodosalicylate (LIS; 0.1 M, pH 8.5). Fractions (800 µl) of

each effluent were collected and samples (80 µl) were used for measurements of radioactivity by liquid scintillation counting. Fractions were also analyzed by SDS-PAGE as described earlier. Protein bands were detected either by autoradiography, or by silver staining using the kit purchased from BioRad. The integrity of the eluted 14S or 5S particles was examined by sucrose gradient centrifugation.

2.12 Interaction of Purified 14S Subviral Particles With Viral RNA

A one ml sample of purified 14S or 5S particles was mixed with different amounts (7-20 µg) of Mengo virus RNA and incubated at room temperature for 90 min. The mixture was then dialyzed against either 0.1 M sodium phosphate buffer (pH 7.2) or 0.01 M Tris buffer with 0.1 M KCl (pH 7.3) at room temperature for 5 hr. The dialyzate was layered onto a 11 ml 15-30% sucrose gradient made in the corresponding sodium phosphate or Tris buffer, and centrifuged to detect the formation of structures larger than the 14S particles.

2.13 Electrophoretic Elution of Viral Structural Proteins from Polyacrylamide Gels

Electroelution of viral structural proteins from SDS-PAGE was carried out as described by Hunkapillar *et al.* (1983). All chemicals were either purchased from the companies suggested by these authors, or the best available

grade was used if the source of the chemicals was not identified. All solutions and gels were made in glass distilled deionized water.

The preparation of phosphate-urea gels (10% acrylamide) was described previously, and 10% Laemmli gels (Laemmli, 1970) were also used. [^{3}H]leucine labelled Mengo virus (~100 µg) was pelleted at 55,000 rpm for 30 min in a SW55Ti rotor. The pellet was then dissolved in appropriate sample buffer and heated to 50-60° for 10-15 min. After electrophoresis, the gel was first fixed in two changes of destaining solution (acetic acid-methanol-water, 50/165/785, v/v/v) for about one hour to remove the urea before staining with 0.5% Coomassie blue in acetic acid-isopropyl alcohol-water (1/3/6, v/v/v). The stained protein bands were cut out and subjected to electroelution. The elution apparatus was fabricated by Dr. J. Tyler (Department of Genetics, University of Alberta) according to the description of Hunkapiller *et al.* (1983). Recovery of proteins was estimated from the recovery of radioactivity, and portions of the eluted samples were analyzed by standard SDS-PAGE and autoradiography in order to establish the identity and purity of the protein(s) contained therein.

2.14 Amino Terminal Sequencing of Viral Capsid Proteins

Electroeluted Mengo viral proteins were subjected to manual micro-sequence analysis as described by Chang *et al.* (1978), ultilizing a coloured Edman reagent

4-N,N-dimethylazobenzene-4'-isothiocyanate (DABITC) for easy identification of the modified amino acid on thin layer chromatographic plates. This method has been reported to be a very sensitive manual sequencing method, only 2-8 nanomoles of peptide or protein being required for a sequence determination up to 20-30 residues.

Automated amino acid sequence determination was carried out by Mr. M. Carpenter using the Applied Biosystems Gas-Phase Sequencer, Model 470A (Hewick *et al.*, 1981). All reagents and solvents were from Applied Biosystems (Foster City, Ca., USA), and polybrene (Klapper *et al.*, 1978) was used as a carrier. Amino acid phenylthiohydantoins were identified by reverse-phase chromatography (Somack, 1980).

2.15 Synthesis and Detection of Acetylated Proteins in the Cell-free System

Three μM [^{3}H]-acetyl CoA (6-7 Ci/mmol) was used as label during a 5 hr incubation of Mengo viral RNA in the rabbit reticulocyte lysate (the volume of incubation mixture was 800 μl). In some experiments unlabelled acetyl CoA was added after 20 min incubation time to a final concentration of 2 mM. At 5 hr, 200 μl of anti-β antibody-conjugated Sepharose was added (in one experiment anti-BSA antibody-Sepharose and BSA , 1.7 ng/ml final concentration, were added to the incubation mixture without RNA as a control), and the sample was mixed on a Adams Nutator (Clay Adams) for 30 min. The antibody-Sepharose was collected by

centrifugation at 12,200 rpm for 10 min in a Beckman Microfuge and the supernatant was removed. One ml of RSB containing 1% NP40 was added, with swirling, to wash away the nonspecifically associated proteins or radioactive materials. After a second wash, 50 µl of 2x gel sample buffer was added to the antibody-Sepharose pellet and the sample was again mixed on the Nutator for 30 min. The antibody-Sepharose was pelleted again and the supernatant containing the extracted capsid proteins was removed and heated in a boiling water bath. Samples were then removed for direct scintillation counting. The incorporation of radioactivity was determined by subtracting the radioactivity of the control sample (no viral RNA added to the translation system) from that of the sample which contained the newly synthesized capsid proteins. These gel samples were also analyzed by standard SDS-PAGE and autoradiography.

3. RESULTS

3.1 Translation of Mengo RNA and Processing of the Viral Polyprotein *in vitro*

3.1.1 Translation of Mengo RNA in Rabbit Reticulocyte Lysates

Mengo virus genome RNA was incubated with nuclease treated rabbit reticulocyte lysate in order to accomplish the cell-free synthesis of viral proteins. The entire genome was successfully translated, as shown by SDS-PAGE analysis of the protein products (Fig. 3.1). The optimal concentration of RNA was found to be 83 µg/ml incubation mixture; higher or lower RNA concentrations resulted in less incorporation of amino acids into proteins. An average of about 6% of the [^{35}S]methionine label (or 7 fold increase over background) was incorporated into newly synthesized proteins during an incubation period of 16 hr. Incorporation of [^{35}S]methionine into EMC viral proteins synthesized in a rabbit reticulocyte lysate was reported to be 45% in 3 hr (Shih *et al.*, 1979); this is much more efficient than the Mengo viral RNA directed *in vitro* translation system described here. Pérez-Bercoff and Gander (1978) have reported an increase of 8-15 times over the background of endogenous activity when Mengo RNA was translated in a lysate of Krebs ascites cells. This is comparable to our results for the *in vitro* translation of Mengo virus RNA in

the rabbit reticulocyte system.

The products obtained by translation of Mengo RNA in the rabbit reticulocyte lysate were analyzed by SDS-PAGE. Fig. 3.1 shows an autoradiograph of a polyacrylamide gel electrophoretic pattern of proteins translated and processed during various time intervals after the addition of Mengo RNA. Lane 10 in Fig. 3.1 shows the pattern obtained with a lysate of Mengo virus infected L-cells. Most of the products from the *in vitro* translation system can be correlated with proteins synthesized in Mengo infected L-cells. Lanes 4a, 6a, 8a and 10 were also scanned for a densitometric comparison of the various protein species (Fig. 3.2), Table 3.1 lists the apparent molecular weights of these proteins, calculated from their electrophoretic mobilities relative to those of marker proteins of known molecular weights.

The time of appearance and disappearance of the various viral protein bands is consistent with the cleavage map shown in Fig. 1.2. The incorporation of [^{35}S]methionine into TCA precipitable products was detectable after 5 min incubation and was found to continue for about one hr. The first virus coded polypeptide to appear was the primary precursor protein designated A1. As shown in Fig. 3.1, lane 3a, A1 was detected by 15 min (0.25 hr) of incubation and was completely processed within 2 hr (lane 6a). The capsid precursors A and B appeared later than A1 (Fig 3.2, 30 min tracing); this was consistent with the role of A1 as

a primary precursor.

Detection of A1 in the cell-free system but not in infected L-cells may indicate that cleavage of A1 in the rabbit reticulocyte lysate is slower than in infected L-cells. Campbell and Jackson (1983) have suggested that, for EMC virus, A1 is cleaved to generate a 'leader peptide' L and the capsid precursor A. When EMC RNA was translated in a reticulocyte lysate system supplemented with unlabelled EMC translation products, A was found to be the major capsid precursor produced during the first 40 min of incubation, and A1 was not detected. If N-formyl-[^{35}S]methionine-tRNA was used in such experiments, the only protein labelled to a significant extent was the small cleavage product L. In the non-supplemented system, this procedure resulted in the labelling of A1. Therefore, it was concluded that, in the presence of the EMC coded proteolytic enzyme, release of L occurred even before synthesis of A1 had been completed. Moreover, since it would be very difficult to stop the translation before any viral protease has been produced in infected cells, it is not surprising that A1 is not normally found in the infected cell lysate.

Protein F, which is encoded in the P2 region of the viral genome, appeared between 15 and 30 min (lane 4a). It reached its maximum level at 2 hr and gradually decreased in amount over the 16 hr incubation period (see Fig. 3.1; also compare the densitometer tracing of Fig 3.1 lane 6a and 8a shown in Fig. 3.2). However, F has always been found to be a

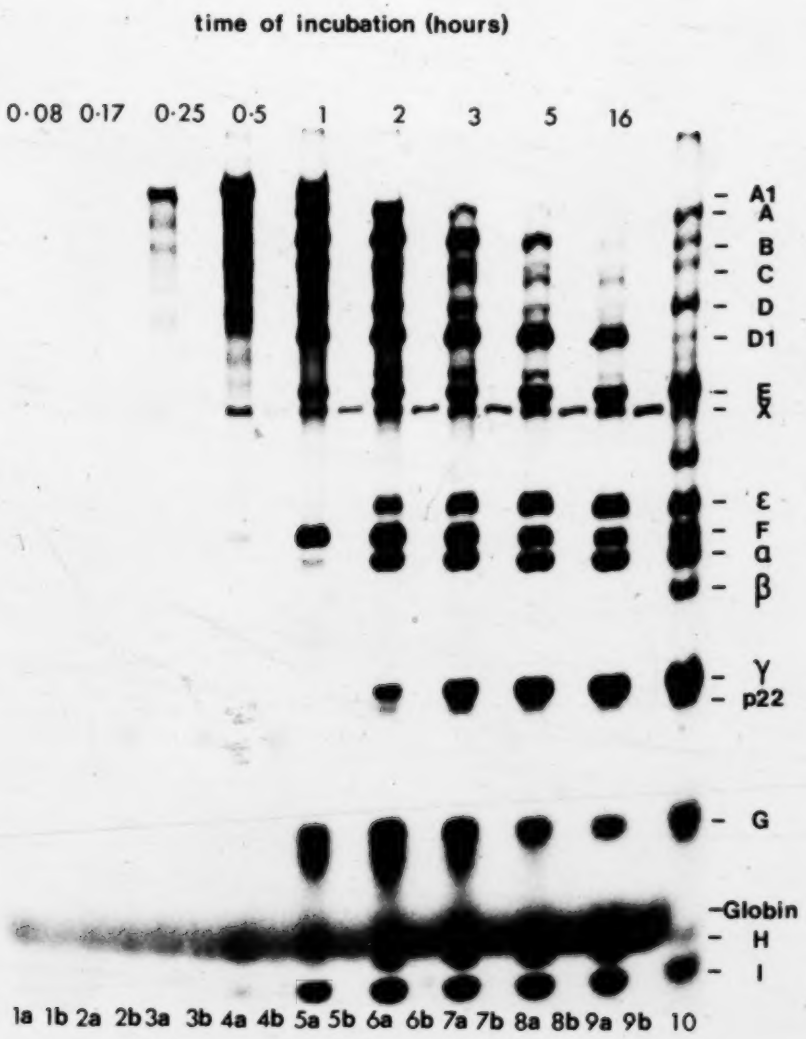

Fig. 3.1 Synthesis of Mengo viral proteins in the rabbit reticulocyte cell-free translation system: analysis by SDS-PAGE and autoradiography.
Standard rabbit reticulocyte protein synthesis mixtures containing [^{35}S]methionine were prepared as described in Materials and Methods. To one mixture (designated 'a' in the figure) was added a suspension of Mengo virion RNA, and to the other (designated 'b') was added an equivalent volume of water. Following incubation for periods ranging from 5 min (0.08 hr; lane 1) to 16 hr (lane 9), samples were removed for analysis by SDS-PAGE and autoradiography as described in Materials and Methods. Lane 10 in the autoradiogram is a reference lysate of Mengo infected L-cells obtained at 6 hr post infection.

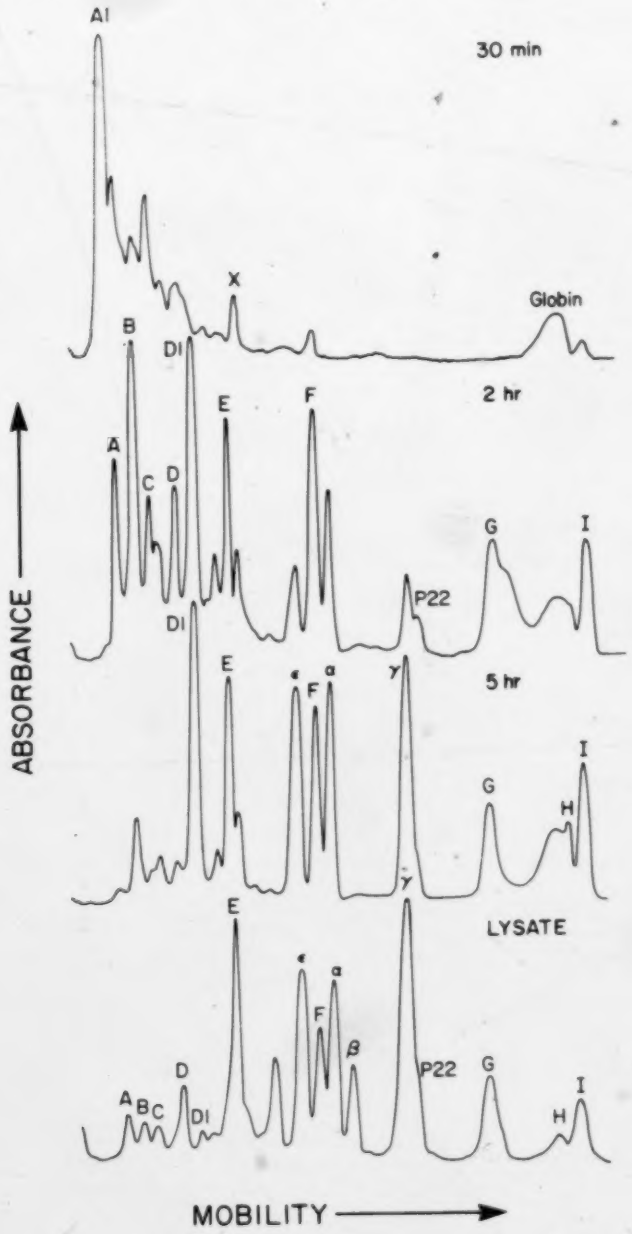

Fig. 3.2 Synthesis of Mengo viral proteins in the rabbit reticulocyte cell-free translation system: densitometer tracings from the autoradiogram of Fig. 3.1.
In order to illustrate the sequential appearance and processing of the Mengo viral proteins in the reticulocyte translation system, lane 4a, 6a, 8a and 10 from the autoradiogram of Fig. 3.1 were subjected to densitometric analysis in the Chromoscan 3.

Table 3.1 Molecular weight estimations from SDS-PAGE.

Polypeptide[1]	Average distance migrated[2] (mm)	Estimated molecular weight[3] (in Kdaltons)
A1	15.0	102.7
A	17.9	98.1
Phosphorylase b[4]	23.8	92.5
B	23.9	89.1
C	28.9	82.2
D	36.8	72.4
Bovine serum albumin[4]	38.6	69.0
D1	43.5	65.1
D2	46.1	62.4
E	55.0	54.1
X	56.3	53.0
Ovalbumin[4]	61.4	46.0
ϵ	74.8	39.4
F	80.4	36.0
α	84.5	33.7
β	91.7	30.0
Carbonic anhydrase[4]	93.4	30.0
γ	109.0	22.8
p22	115.4	20.6
Lactoglobulin A[4]	123.2	18.4
G	132.0	15.8
Globin	149.5	11.9
H	158.1	10.4
I	163.2	9.6
δ	173.9	8.1

[1]Taken from SDS-PAGE analysis of infected L-cell lysates (6 hr p.i.) and Mengo RNA programmed reticulocyte lysates which included marker proteins.
[2]Average of two determinations.
[3]Calculated from a standard curve of logMW versus distance migrated (Shapiro *et al.*, 1967).
[4]Polypeptide markers used to construct the standard curve.

stable final translation product in Mengo, EMC or poliovirus infected cells, and in the EMC or polio messenger RNA directed cell-free system (Butterworth and Rueckert, 1972b; Lucas-Lenard, 1974; Paucha *et al.*, 1974; Shih *et al.*, 1978; Shih *et al.*, 1979). It is possible that protein F of Mengo virus can be degraded slowly in the rabbit reticulocyte lysate system by endogenous proteases.

Precursor protein C, which is encoded near the 3'-end of the viral RNA, also appeared at about the same time as F (Fig. 3.1, lane 4a; Fig 3.2, 30 min tracing). It was processed rapidly into intermediate D and then into the polymerase E and the putative protease p22.

After about 30 min to 1 hr, a protein band identified as D1 on the basis of its apparent molecular weight, appeared together with structural protein α and nonstructural protein E (Fig. 3.1, lane 5a). These are secondary cleavage products produced by the viral protease (Palmenberg *et al.*, 1979; Palmenberg and Rueckert, 1982). The Mengo structural protein intermediate D2, which was believed to be the cleavage product of B and the immediate precursor of capsid proteins α and γ in infected L-cells (Paucha *et al.*, 1974), was not found in the protein products of cell-free synthesis. Moreover, the structural proteins ε and γ appeared together after about 2 hr incubation time, following the appearance of protein α (Fig. 3.2). These observations lead to the conclusion that (similar to the situation in an EMC RNA directed cell-free system;

Shih *et al.*, 1979) the Mengo protein precursor B was cleaved into D1 and α, and D1 was then cleaved into ϵ and γ in the cell-free system. No further processing of ϵ to β and δ was observed. The latter observation implies that maturation of Mengo virion does not occur in this rabbit reticulocyte system during the 16 hr incubation period.

After 16 hr of incubation, the major viral protein bands that remained were D1, E, ϵ, F, α, γ, G, H, and I (Fig. 3.1, lane 9a). Almost all of the precursors A1, A and B had been processed by 16 hr, but cleavage of D1 seemed to be relatively slow, as was found during the cell-free synthesis of EMC viral proteins (Shih *et al.*, 1979). Processing of viral proteins in infected L-cells is virtually completed within 8-10 hr post infection (unpublished observations) and is always accompanied by the cleavage of ϵ and formation of progeny virions. Therefore, it seems possible that cleavage of D1 might not be completed until the ϵ product is further processed into β and δ.

Although most of the protein bands can be identified as viral proteins, there are still some whose origin and function are not defined. One of them is a protein of apparent MW 60,000 which was located between D1 and E in the polyacrylamide gel and was visible in most of the later time samples. Although it corresponds to a minor band in the infected cell lysate shown in lane 10 of Fig 3.1, its identity is not known. Another prominent protein band in the infected L-cell lysate, having a MW of about 47,000, was

found to be only a minor band throughout the 16 hr incubation time in the *in vitro* translation system. In the case of EMC virus infected cells, it has been suggested that this is a host protein (Butterworth and Rueckert, 1972b).

Nonviral radioactive proteins with apparent MW's of 53,000 (protein X) and 12,000 were also detected in incubation mixtures with and without added Mengo RNA (Fig. 3.1). The protein X band probably resulted from mRNA-independent labelling of a pre-existing protein, since it was not visible in autoradiograms if [^{3}H]leucine was used to label the *in vitro* translation products (result not shown; Pelham and Jackson, 1976). Jackson and Hunt (1983) also found that this protein X band migrated with an apparent MW of 220,000 if the sample was not boiled prior to SDS-PAGE. Since the protein X band did not obscure the Mengo protein pattern, the usual boiling of samples was continued. The diffuse band at about 12,000 MW was globin, the dissociated α and β subunits produced from the reticulocyte hemoglobin by boiling in gel sample buffer (Jackson and Hunt, 1983); this represented about 90% of the total protein in the rabbit reticulocyte lysate. Another band at around 20,000 MW was found in the control incubation mixtures during early incubation time (Fig. 3.1, lane 1b, 2b, 3b and 4b) and disappeared after incubation for 2 hr or more (lane 6b - 9b). Jackson and Hunt (1983) suggested that this was the peptidyl-tRNA resulting from translation of fragments of globin mRNA, and could be removed by treating

the incubation mixture with RNase (50-100 µg/ml) prior to electrophoresis.

3.1.2 Translation of Mengo RNA in Wheat Germ Cell-free Extracts

Mengo virus genome RNA was also incubated with a nuclease treated wheat germ system. The incorporation of [^{35}S]methionine was found to be as low as 1% of the added label. Wheat germ translation mixtures with and without added Mengo RNA were analyzed by SDS-PAGE. A number of high molecular weight bands were found in both incubation mixtures. Some high MW bands as well as one or two low MW bands were found only in the sample with the Mengo RNA. However, the molecular weights of most of these protein bands did not correspond to known Mengo virus proteins. It is possible that these polypeptides were produced by the premature termination of protein synthesis. Accumulation of incomplete translation products has been found in the wheat germ cell-free system programmed with tobacco mosaic virus RNA (Abraham and Pihl, 1980) or with avian vitellogenin mRNA (Gordon *et al.*, 1977).

The possibility of premature termination could be tested, since the resulting truncated viral precursor proteins should still be processed if the protease were present. Therefore, an unlabelled 5 hr incubation mixture of Mengo RNA programmed rabbit reticulocyte lysate (containing the p22 protease) was added to a wheat germ extract which

had been programmed with Mengo RNA and incubated for 5 hr in the presence of [^{35}S]methionine. This mixture was then incubated for an additional 5 hr. The protein pattern revealed by SDS-PAGE and autoradiography did not show remarkable changes except for the appearance of one band at an apparent MW of 70,000 which did not correspond to any of the Mengo viral proteins already identified. This result might indicate that the protein bands observed in the Mengo RNA programmed wheat germ translation system were not authentic viral proteins (a consequence of random initiation and/or termination due to misreading of the RNA codons), or that the truncated viral precursors were not in the proper conformations for recognition and cleavage by the viral protease.

An alternative explanation might be that endogenous nucleolytic activities may have caused hydrolysis of the viral RNA at a limited number of preferentially sensitive sites. However, addition of the human placental ribonuclease inhibitor, RNasin (1 unit/μl incubation mixtures), did not alter the extent of protein synthesis or the polypeptide pattern in the wheat germ system.

Since the 5'-end of Mengo virus RNA is covalently linked to the VPg protein instead of being capped, proper translation of this messenger in the wheat germ system may not occur if the enzyme responsible for the removal of VPg is absent. However, Cowpea mosaic virus RNA molecules, which also contain 5'-VPg's, have been successfully translated in

the wheat germ cell-free system (Goldbach *et al.*, 1981; Franssen *et al.*, 1982).

The reasons for the poor performance of Mengo RNA in the wheat germ system are not clear.

3.2 Production of Viral 14S Particles

3.2.1 Production of Viral 14S Particles in the Cell-free Translation System

Since the synthesis and processing of Mengo viral proteins in the rabbit reticulocyte lysate had proven to be successful, the possibility that subviral structures containing the capsid proteins or their precursors might assemble in this system was investigated. To detect any 5S, 14S or other particles which might be produced, *in vitro* translation mixtures (containing [^{35}S]methionine) with or without Mengo RNA were incubated for 1 hr, 5 hr, 16 hr and 32 hr and then centrifuged in 5-20% sucrose gradients. Gradients were fractionated, and the TCA precipitable radioactivities were measured. Fig. 3.3 shows the profiles of these gradients.

After a 1 hr incubation, most of the radioactively labelled material synthesized in the cell-free system sedimented in the 5S region (as judged from proximity to a 4.2S hemoglobin marker; Chiancone *et al.*, 1966). SDS-PAGE analysis of this region showed that the percursor proteins A and B had already been cleaved, and that their proteolytic

products D1, ϵ, α and γ were the predominant components of the 5S peak (Fig. 3.4, panel A, lane 2). As shown in Fig. 3.3, panel A, a comparatively small peak was present in the 14S region after only 1 hr incubation. SDS-PAGE analysis showed that both precursor A and its cleavage products ϵ, α and γ existed in this 14S peak (Fig. 3.4, panel B, lane 2), which probably comprised mixtures of premature ($[A]_5$) and mature ($[\epsilon\alpha\gamma]_5$) pentamers. The 5S particles ($[\epsilon\alpha\gamma]$) did not seem to be degradation products of the 14S particles since the direction of transfer of radioactivity was from the 5S peak to the 14S peak during the course of a 16 hr incubation period (Fig. 3.3). Thus, it would appear that the rabbit reticulocyte lysate system not only translates the Mengo virus RNA faithfully, but also provides the necessary factors and conditions for the assembly of 14S subviral particles. Since the amount of the radioactively labelled 14S material ceased to increase after 16 hr and the major components of the 14S peak were found to be ϵ, α and γ, this time was used in all subsequent studies involving the production of 14S particles in the reticulocyte lysate (Fig 3.3 and 3.4). No structures larger than 14S were found by sucrose gradient centrifugation of programmed reticulocyte lysates (even if these were incubated for more than 16 hr).

To serve as controls, incubation mixtures without addition of any messenger RNA were also centrifuged in 5-20% sucrose gradients. As shown in Fig. 3.3 (open circles),

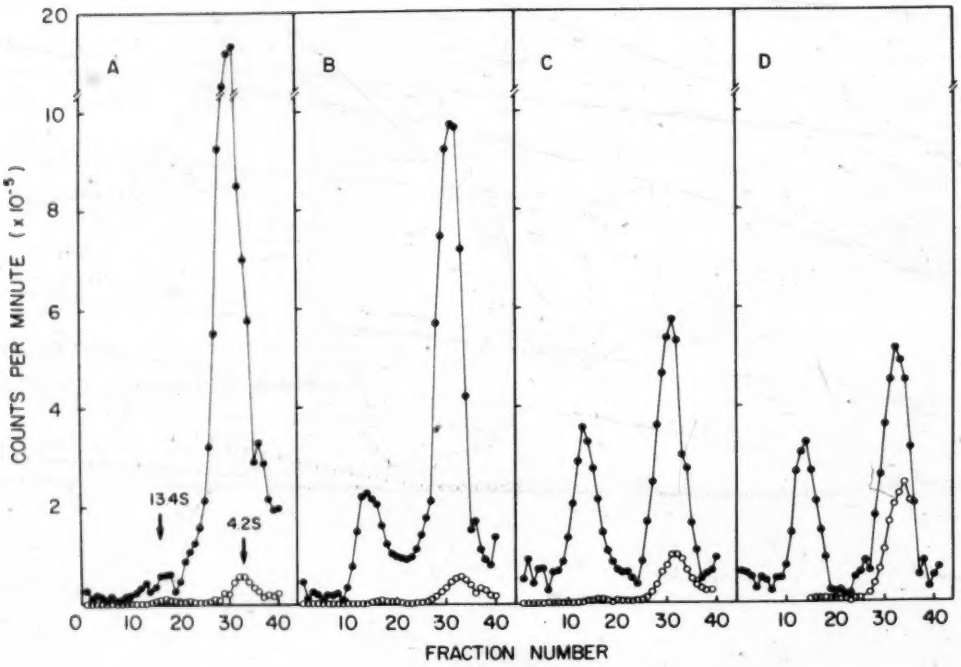

Fig. 3.3 Sucrose density gradient centrifugation profiles of the Mengo virus protein structures formed during incubation in the *In vitro* translation system.
Reticulocyte lysates containing [^{35}S]methionine were incubated in the presence of Mengo RNA or in its absence for various periods of time. Samples were then collected and centrifuged in sucrose density gradients as described in Materials and Methods. [^{3}H]leucine labelled 13.4S Mengo virion subunits were centrifuged in parallel to provide a sedimentation marker; and the 4.2S hemoglobin (Chiancone *et al.*, 1966) in the rabbit reticulocyte lysate itself served as an internal marker. Panels A, B, C and D represent samples taken after incubation for 1, 5, 16 and 32 hr, respectively. The [^{35}S] radioactivity is expressed in counts per min per gradient fraction (270 µl) for samples from lysates with added Mengo RNA (•-•) or without (o-o).

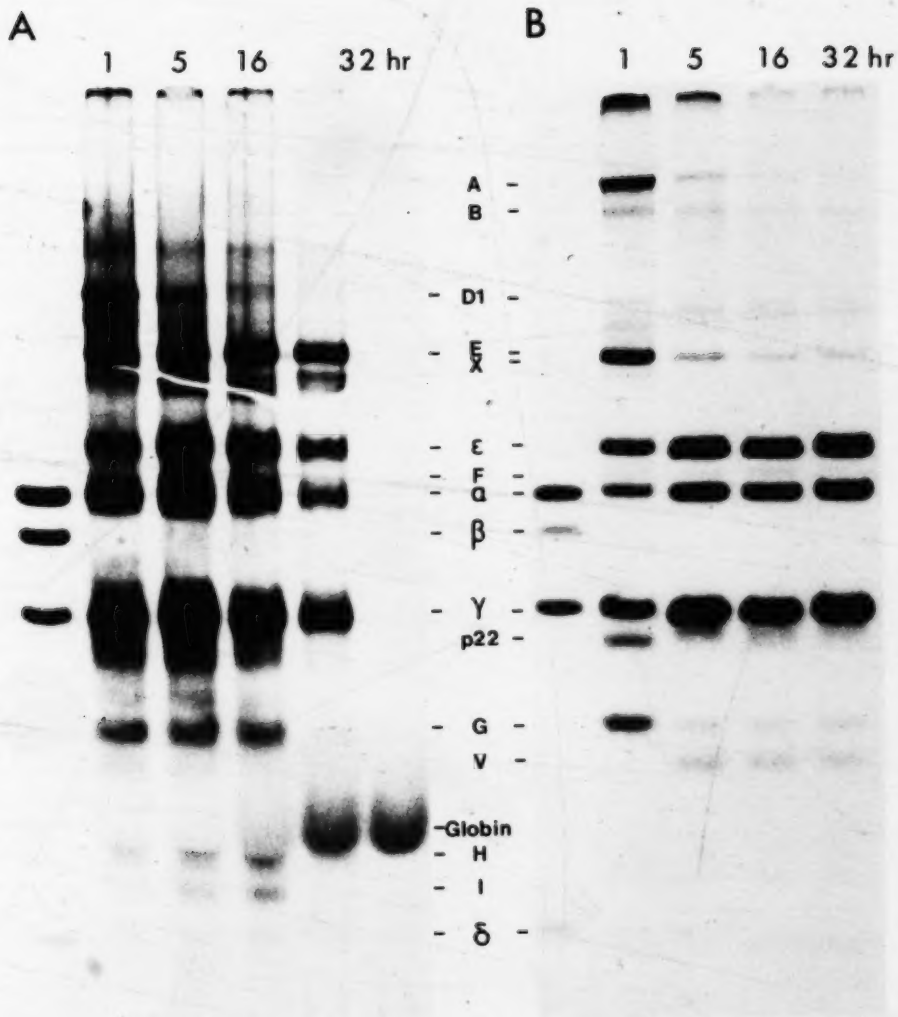

Fig. 3.4 SDS-PAGE analysis of the 14S and 5S peak fractions obtained from sucrose density gradient centrifugation of Mengo RNA programmed reticulocyte translation mixtures.
Panel A shows the SDS-PAGE autoradiogram of the 5S peaks obtained after sucrose density gradient centrifugation of the reticulocyte lysates containing Mengo RNA (see Fig. 3.3); panel B shows a similar autoradiogram of the 14S peak material. Lane 1 in both panels shows the band pattern of [^{3}H]leucine labelled Mengo virions and lanes 2, 3, 4 and 5 represent incubation times of 1, 5, 16 and 32 hr, respectively, for the *in vitro* protein synthesis system. Lane 6 in panel A is the 5S peak material from a control incubation mixture after sucrose gradient centrifugation. Except for the marker Mengo virion and the sample in lane 6 of panel A, 1.2×10^5 cpm of [^{35}S] were loaded onto each lane of panel A and 6.6×10^4 cpm of [^{35}S] onto each lane of panel B.

radioactive materials from unprogrammed lysates also sedimented in the 5S and 14S regions. SDS-PAGE analysis showed that the '5S' material was predominantly globin, which corresponded to the 4.2S hemoglobin marker present in the reticulocyte lysate (Fig. 3.4, panel A, lane 6). Protein X was found to sediment slightly slower than the viral 14S particle as a stable structure. It could withstand treatment with NP40, EDTA, RNase or mercaptoethanol, and prolonged dialysis against RSB, and still retain its integrity during recentrifugation. However, the amount of radioactive label found in this '14S' peak varied with different batches of lysate. In more recent *in vitro* translation experiments (for example the gradient shown in panel B of Fig. 3.5), protein X and its '14S' aggregate were present only as minor contaminants.

Viral 14S particles from the reticulocyte lysate were also prepared according to the procedure of Palmenberg (1982), wherein a 16 hr incubation mixture was digested with RNase and diluted with an equal volume of 20 mM HEPES buffer (pH 6.9), containing 200 mM KCl, 5 mM $MgCl_2$ and 5 mM 2-mercaptoethanol (this buffer replaces RSB). The sample was then layered onto an 11 ml, 5-20% sucrose gradient prepared in the same buffer. The resulting 14S peak was analyzed by SDS-PAGE. No significant difference in terms of composition or yield was observed between the 14S particles prepared by this and the previously described method.

3.2.2 Comparison of the 14S Particles Produced in Infected L-cells and in the Cell-free Translation System

Proteins which sedimented at various positions in the gradient obtained from a 16 hr incubation of the programmed reticulocyte lysate were analyzed by SDS-PAGE, and Fig. 3.5, panel B, shows the autoradiogram of the polyacrylamide gel. Many viral proteins were found throughout the gradient. The nonstructural proteins E and G were enriched in fractions near the top of the gradient, whilst the majority of the capsid proteins ϵ, α and γ were found in fractions 11-16 and 27-34. These fractions correspond to 14S and 5S peak regions, respectively. An unknown protein designated V was sometimes found in the 14S region (see Fig. 3.7, lane 7). V migrated slightly faster than the viral protein G in SDS-PAGE, and was not present in the programmed reticulocyte lysate prior to centrifugation (see, for example, Fig. 3.7, lane 8). It could conceivably be a product of one of the viral proteins which was cleaved during sucrose gradient centrifugation. The recovery of labelled proteins from the 5S region presented a problem, because TCA-acetone treatment of these hemoglobin containing fractions produced a dark red pellet which did not dissolve in gel sample buffer even after boiling. Since the recovery of radioactivity was not quantitative, the apparent absence of the large precursor molecules in this region may be illusory.

Isolation of viral 14S particles from infected L-cells was done in collaboration with Dr. Ulrike Boege, following

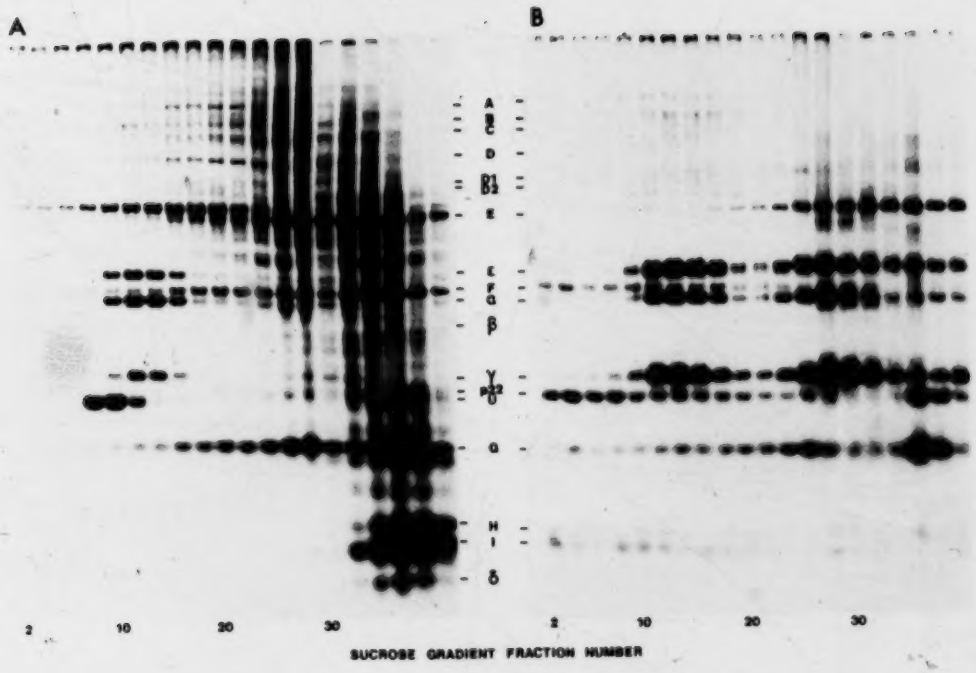

Fig. 3.5 Distributions in sucrose gradients of [^{35}S]methionine labelled polypeptides synthesized under the direction of Mengo RNA *in vivo* and *in vitro*.
A lysate of Mengo virus infected L-cells obtained at 4 hr post infection was subjected to sucrose density gradient centrifugation as described in Materials and Methods. Consecutive pairs of fractions were combined and analyzed individually for protein composition by SDS-PAGE and autoradiography. The results are shown in panel A. Panel B shows the results of a similar analysis of a standard reticulocyte lysate translation mixture which was incubated with Mengo RNA for for 16 hr. Direction of sedimentation is from right to left.

the protocol described in Materials and Methods. The radioactivity profile obtained from sucrose density gradient centrifugation of a lysate of Mengo-infected L-cells ([^{35}S]methionine; 4 hr p.i.) was very similar to that obtained with the Mengo RNA programmed reticulocyte lysate (16 hr incubation; see above). Fig. 3.5, panel A, shows the autoradiogram of the SDS-PAGE analysis of the sucrose gradient fractions obtained from a lysate of infected L-cells. In this case, the 14S region contained most of the capsid proteins ϵ, α and γ, and minor amounts of A, B, C, D, E, F and G. A radioactively labelled cellular protein designated U, present in the sucrose gradient of the mock infected cell lysate (data not shown), was also found in the L-cell lysate. Protein U is similar to the rabbit reticulocyte protein X (discussed earlier) in its ability to form a stable particle which sediments at or near the 14S region.

The distribution of viral proteins following sucrose density gradient centrifugation of the infected L-cell lysate was somewhat different from that obtained with the programmed reticulocyte lysate, as can be seen by comparing panels A and B in Fig. 3.5. One difference is the localization of the smaller non-structural proteins G, H and I near the top of the gradient in the case of the infected L-cell lysate. This might simply reflect the dissociation of non-specific aggregates of these proteins by the DOC and NP40 (the final concentration of each was 1%) which were

added to the L-cell lysate before centrifugation. Secondly, the bulk of the capsid proteins and their precursors were found in the 14S rather than the 5S region of the gradient obtained from the infected L-cells. In the reticulocyte lysate, on the other hand, larger amounts of these proteins were found in the 5S region. Assuming that the 14S particles assemble via the same pathway in both systems, it would seem that the rate of formation of 14S pentamers from 5S monomers was facilitated *in vivo*. This might imply, in turn, that a morphogenetic factor present in L-cells facilitated this process.

Viral 14S particles prepared from both the *in vivo* and *in vitro* systems could be recovered from the sucrose gradient fractions by dialysis against RSB at 4°, provided that the dialysis bags had previously been boiled with sodium bicarbonate and EDTA. When the dialyzates were recentrifuged, 14S peaks could be recovered from both the *in vivo* and *in vitro* materials. Thus, the sedimentation behaviour of the 14S particles produced in infected L-cells were identical to those of the 14S particles obtained from the programmed reticulocyte lysate.

3.3 Purification of the 14S Subviral Particles Obtained from Cell-free Translation

3.3.1 Immunoaffinity Column Chromatography

Gradient fractions containing the 14S peak material from the *in vitro* translation system were combined and loaded onto an immunoaffinity column (50 µl bed volume) containing anti-β IgG, and prepared as described in Materials and Methods. The results of the sequential elutions from the column are summarized in Table 3.2 and Fig. 3.6. About 75% of the 14S peak material from the sucrose gradients was bound to the column, as judged from the disappearance of radioactivity after loading and washing with RSB containing 1% NP40, and then RSB alone. The major component of the unbound material was the rabbit reticulocyte protein X (identified by SDS-PAGE analysis), but some of the structural proteins (ϵ, α and γ) also passed through the column (probably due to its relatively small capacity). As expected, the trace amounts of the nonstructural proteins which were present in the original 14S peaks were not bound to the immunoaffinity column. The flow-through material sedimented in the 14S and 5S regions when recentrifugated in a sucrose gradient; the 14S peak contained mostly protein X (Fig. 3.6, panel A; Table 3.2).

A variety of conditions and chemicals (including acid pH, alkaline pH and different salt solutions) were tested for their ability to elute ϵ, α and γ from the immunoaffinity column. The only reagent listed by MacSween and Eastwood (1981) which worked in this regard was the strongly chaotropic salt, lithium diiodosalicylate (LIS).

Table 3.2 Purification of *in vitro* viral 14S particles by immunoaffinity chromatography.

Column effluent	Recovery[1]		Protein[2] detected in autoradiograph	Particles identified by recentrifugation
	Exp.1	Exp.2		
Flow through during loading	13.8%	20.1%	X, ε, α, γ, (F, p22, G)	14S(mostly X), ~5S(ε, α, γ and some nonstructural proteins)
Washings with RSB and RSB+1%NP40	9.4%	4.6%	X, ε, α, γ, (F, p22, G)	14S(X) and ~5S(ε, α, γ and some nonstructural proteins)
Displacement by Mengo 13.4S virion subunits	6.6%	8.5%	ε, α, γ, (D1, V)	14S(ε, α, γ)
Elution with LIS[3]	42.5%	35.7%	ε, α, γ, (V, p22)	5S(ε, α, γ)
1% SDS wash	2.1%	0.8%	ε, γ, G, (α, F, p22, V, H)	-
Gel bed	-	1.1%	-	-

[1]The total amount of radioactivity applied to the column in each experiment was taken to be 100%.
[2]Proteins present in minor or trace amounts are indicated in parentheses.
[3]Lithium diiodosalicylate.

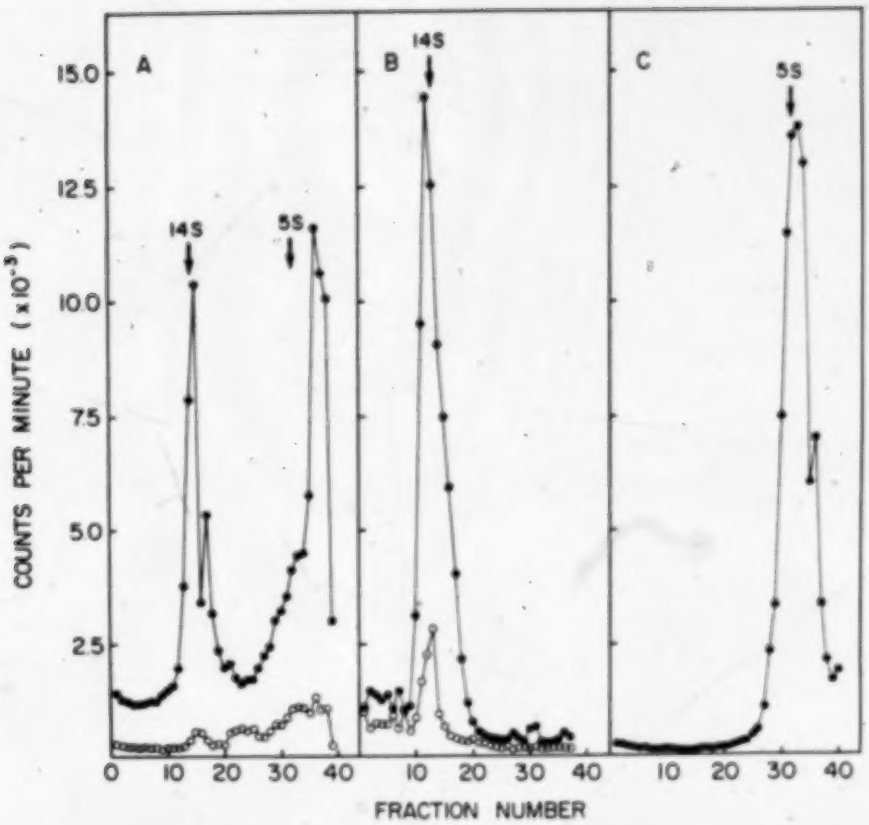

Fig. 3.6 Immunoaffinity chromatography of Mengo 14S particles produced in the *in vitro* translation system: sucrose density gradient centrifugation of the immunoaffinity column effluents.
[^{35}S]methionine labelled Mengo 14S particles produced during a 16 hr incubation in the viral RNA programmed reticulocyte lysate were collected by sucrose gradient centrifugation (see Fig. 3.3). An immunoaffinity column containing anti-β IgG was prepared and chromatographic procedures were carried out as described in Materials and Methods. Column effluents were centrifuged in 5-20% sucrose gradients and the fractions obtained were analyzed for radioactivity. Panel A shows the sedimentation profile of the flow-through material obtained during sample loading (•-•) and that of the material washed out of the column by RSB containing 1% NP40 (o-o). Panel B shows the profile of radioactive material displaced from the column by unlabelled 13.4S virus capsid subunits; one sucrose gradient was prepared in RSB (o-o), as were those for the experiments shown in panels A and C, while the other was prepared in 0.1 M sodium phosphate, pH 7.2, containing 1% NP40 (•-•). Panel C shows the sedimentation profile of material subsequently eluted from the immunoaffinity column by lithium diiodosalicylate (LIS).

LIS was, however, not suitable for eluting 14S particles from the immunoaffinity column because it disrupted protomer-protomer interactions as well as antibody-antigen interactions, thereby producing an effluent of [$\epsilon\alpha\gamma$] monomers which sedimented at ~5S (Fig. 3.6, panel C).

To elute the specifically bound 14S particles from the column in an intact form, 13.4S virion capsid subunits were employed as agents to competitively displace the 14S particles from the antibody binding sites. The column was subjected to gradual equilibration with the 13.4S subunits (in RSB), and elution was carried out by the continued addition of the subunit suspension. The radioactivity displaced from the column quickly reached a plateau level which was maintained until about 25 fractions (20 ml) had been collected. Approximately 10% of the total bound radioactivity was recovered by the 'homochromatography' procedure (Table 3.2). When LIS was used as an eluting agent following the 'homochromatography' with 13.4S subunits, about 50% of the radioactivity which had remained bound was removed from the column. As expected, this material sedimented as 5S monomers (protomers), and was composed of approximately equimolar amounts of ϵ, α and γ (see below). Minor contamination with V and p22 was occasionally detected.

As shown in Fig. 3.6, panel B, the radioactive material displaced from the column by 13.4S subunits sedimented at ~14S in 5-20% sucrose gradients with no indication of

dissociation into 5S products. However, approximately 90% of the added radioactivity in this gradient was found as a pellet at the bottom of the centrifuge tube. Recovery of purified 14S particles was substantially improved by the addition of detergent (1% NP40) to both the sample and the gradient. As shown in panel B of Fig. 3.6, centrifugation of the 14S effluent in a detergent containing sucrose gradient made in 0.1 M sodium phosphate, pH 7.2 (filled circles), resulted in a much greater yield of 14S particles than when the column effluent was centrifuged in a sucrose gradient made in RSB (open circles). Recentrifugation of the column effluent in a sucrose gradient made in RSB, but containing 1% NP40, revealed a similar influence of the detergent on the recovery of 14S particles. It seems that the purified pentamers recovered from the immunoaffinity column have a strong tendency to aggregate and are pelleted during subsequent centrifugation unless detergent is present to suppress hydrophobic pentamer-pentamer interactions.

The 14S subviral particles made in infected L-cells and isolated by sucrose gradient centrifugation were also applied to the immunoaffinity column. In this case the percentage of the total radioactivity which was bound was only about 34% (compared to the retention of 75% of the applied 14S particles from the *in vitro* translation system). This difference could be accounted for by the fact that appreciable amounts of cellular proteins (in particular, protein U) continued to be synthesized in the virus-infected

cells. These proteins, like X, were not retained by the anti-β antibodies in the column. A second difference in elution behaviour was that the relative amounts (percent of the total starting material) of radioactive ϵ, α and γ displaced by the 13.4S subunits and eluted with LIS were roughly equal (8% and 11%, respectively, of the total radioactivity loaded onto the column) for the 14S particles produced *in vivo*; whereas the ratio of these amounts was about 1:5 for those produced by *in vitro* translation (Table 3.2). This result implies that there are conformational differences between the 14S particles obtained from the two sources, even though both were recognized by the same monoclonal antibody.

3.3.2 Assessment of the Purity of the 14S Particles Obtained by Immunoaffinity Chromatography

Samples obtained during purification of 14S particles produced from infected cells and from rabbit reticulocyte lysates were analyzed by SDS-PAGE, and the autoradiogram is presented in Fig. 3.7. Lanes 2 and 8 show the starting materials for preparations of 14S particles, namely the infected L-cell lysate (4 hr p.i.), and the Mengo RNA programmed reticulocyte lysate (incubated for 16 hr), respectively. The 14S peaks isolated from 5-20% sucrose gradients of the infected L-cell lysate and the Mengo RNA programmed reticulocyte lysate are shown in lanes 3 and lane 7, respectively. As mentioned above, more structural

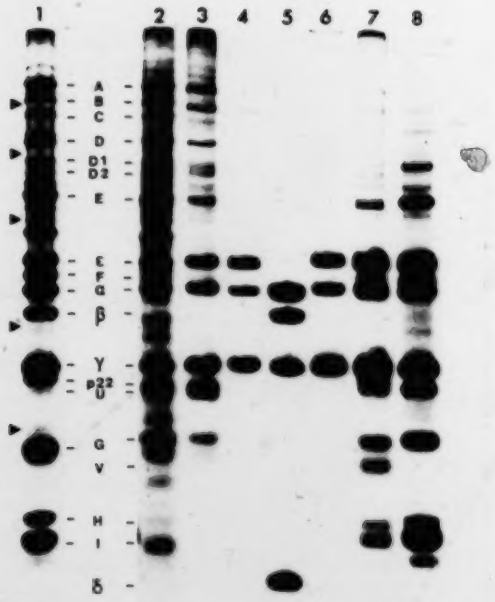

Fig. 3.7 Purification of the 14S particles from Mengo virus infected L-cells and from the Mengo RNA programmed reticulocyte lysate: analysis of [^{35}S]methionine labelled proteins by SDS-PAGE and autoradiography.
Lane 1 of the autoradiogram shows the profile of radioactive proteins in a lysate of Mengo infected L-cells 6 hr post infection. By this time, progeny virion production has commenced and the accompanying morphogenetic cleavage of ϵ to β+δ is taking place (the δ polypeptides are barely visible in the autoradiogram since they each contain only one methionine residue). Lane 2 shows the protein profile from infected L-cells 4 hr post infection, and before progeny virion assembly has begun. This represents the starting radioactive material for the purification process. The starting material from the *in vitro* translation system is represented by lane 8 which shows the radioactive protein profile of the Mengo RNA progammed reticulocyte lysate after 16 hr incubation. Lanes 3 and 7 represent the 14S peaks obtained by sucrose density gradient centrifugation of the *in vivo* and *in vitro* lysates, respectively. Lanes 4 and 6 show the radioactive protein profiles of the 14S particles displaced from the immunoaffinity column by unlabelled 13.4S Mengo capsid subunits (lane 4 shows the particles from infected L-cells and lane 6 shows those from the programmed reticulocyte lysate). Lane 5 shows the protein profile of [^{3}H]leucine labelled Mengo virions. [^{14}C]amino acid labelled molecular weight markers were electrophoresed concurrently; these are indicated by the arrowheads on the left and were (from top to bottom) phosphorylase b (MW 92,500), bovine serum albumin (69,000), ovalbumin (46,000), carbonic anhydrase (30,000) and lactoglobulin A (18,500).

precursor proteins are present in the 14S peak from the infected L-cells while more nonstructural proteins are present in the 14S peak from the *in vitro* system. Immunoaffinity column chromatography removed most of the precursors and nonstructural proteins, as shown in lane 4 and 6. Occasionally small amounts of protein V were detected with the eluted 14S particles from the *in vitro* system, otherwise these particles contained only radioactive ϵ, α and γ in roughly equimolar amounts.

The purity of the 14S particles following sucrose gradient centrifugation and immunoaffinity chromatography has been discussed in terms of the radioactively labelled proteins. However, it is to be expected that unlabelled cellular or rabbit reticulocyte proteins would be present in significant amounts in the original lysates. As shown by the total protein content analysis in Fig. 3.8, both the 16 hr incubation mixture from a reticulocyte lysate (lane 1) and the infected L-cell lysate at 4 hr p.i. (lane 2) contain amounts of unlabelled proteins to the extent that the newly synthesized Mengo viral proteins cannot be identified above background (compare lane 2 with lane 3 - the uninfected L-cell lysate).

Sucrose density gradient centrifugation removed the bulk of the reticulocyte proteins, especially globin, from the 14S peak material produced from the cell-free translation system (lane 4). However, the same treatment was not sufficient to remove the high molecular weight cellular

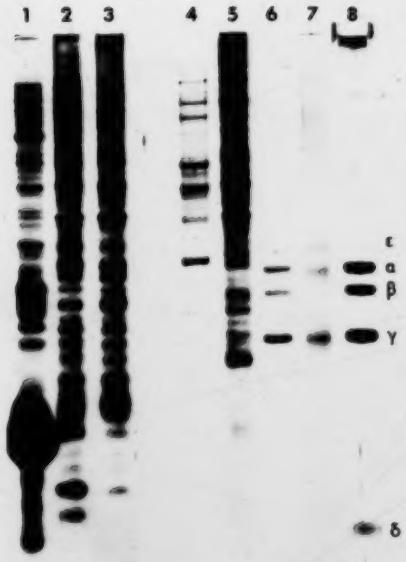

Fig. 3.8 Purification of the 14S particles from Mengo virus infected L-cells and from the Mengo RNA programmed reticulocyte lysate: analysis of total protein composition by SDS-PAGE and silver staining.
Samples were prepared and subjected to SDS-PAGE as described in Materials and Methods. Following electrophoresis, the gels were stained by forming protein-silver complexes using the reagent kit supplied by BioRad Laboratories. The amount of protein applied to each lane was determined by a fluorescamine method (Boehlen *et al.*, 1973), and the percentage of the total amount of protein from the particular purification step which this represents was calculated; these values are given below in parentheses. Lane 1 shows the total protein complement of the Mengo RNA programmed rabbit reticulocyte lysate incubated for 16 hr (50 μg, 1%). Lane 2 shows the proteins in Mengo virus infected L-cells at 4 hr post infection (30 μg; 0.1%), while lane 3 shows those in a lysate of uninfected L-cells (30 μg, 0.1%). Lane 4 shows the proteins in the 14S peak from sucrose gradient centrifugation of the reticulocyte lysate after incubation for 16 hr with Mengo RNA (15 μg; 12%), and lane 5 shows the 14S peak from the infected L-cells (18 μg; 5%). Lane 6 shows the proteins obtained when 14S particles from L-cells were displaced from the immunoaffinity column by 13.4S capsid subunits (4 μg; 5%), and lane 7 shows the proteins eluted from the column with LIS (4 μg; 50%). Lane 8 represents the proteins in purified Mengo virions (10 μg). The development times for the silver staining were 15 min for lanes 1-3 and 30 min for lanes 4-8.

proteins from the 14S material produced *in vivo* (lane 5). It was expected that the immunoaffinity chromatography and elution of the 14S particles by 13.4S viral subunits would remove the cellular proteins. However, because of the very small amounts of proteins synthesized in the rabbit reticulocyte lysates, the total amount of 14S proteins recovered from any particular column was only about 1 picomole. Silver staining revealed, therefore, only the α, β and γ proteins of the 13.4S subunits (data not shown); this was also true for the 14S material obtained from infected cells (lane 6). In view of this limitation, it was reasoned that the purity of the 14S particles *vis á vis* the L-cell or reticulocyte lysate starting materials could be inferred by ascertaining the total protein composition of the 5S particles eluted from the column by LIS. As shown in Fig. 3.8, lane 7, the 14S particles from infected L-cells eluted by LIS contained ϵ, α and γ. The only contaminants observed were trace amounts of the precursor of ϵ and γ (*i.e.* D1) and a protein of MW 38,000, which appeared to be of cellular origin since it was not radioactively labelled. Since it is reasonable to assume that LIS might dislodge contaminants from the immunoaffinity column at least as effectively as would the 13.4S subunits, it was concluded that the displaced 14S particles were essentially pure virus-specific structures. It is, however, impossible to estimate the degree of purification in quantitative terms, since the amount of 14S particles in the starting material

cannot be determined.

3.3.3 Storage of the 14S Particles

In order to be able to accumulate 14S particles for later assembly studies, it was necessary to find conditions for storage of these entities. Purified 14S particles obtained from immunoaffinity chromatography were centrifuged into a cushion of 70% glycerol or of 60% sucrose in RSB. Although we had successfully concentrated most of the 14S material into the 60% sucrose cushion, 90% was lost during subsequent dialysis. The particles concentrated into 70% glycerol were stored at -20°. When these were later dialyzed and recentrifuged in 5-20% sucrose gradients (in RSB containing 1% NP40), 14S peaks were observed. The percentage of the radioactivity in the glycerol cushion which was recovered in the peak was about 30%. Since these purified 14S particles had strong tendencies to aggregate and might be pelleted during centrifugation, addition of 1% NP40 into the sucrose or glycerol cushion, as well as the dialysis buffer, might improve the recovery of the 14S particles.

3.4 Interaction of the 14S Particles with Viral RNA

Since the 14S pentamers are believed to be the fundamental structural components for virion assembly, preliminary studies were carried out in order to examine the interaction of purified 14S particles with isolated Mengo RNA. Radioactively labelled 14S particles prepared by

in vitro translation and purified as described above were incubated alone or with 7 μg or 20 μg of Mengo RNA, in RSB, at room temperature for 90 min. The mixture was then dialyzed against either 0.1 M KCl, 0.01 M Tris (pH 7.3) or 0.1 M sodium phosphate (pH 7.2) for 5 hr at room temperature. To detect the formation of large particles, dialysates were centrifuged in 15-30% sucrose gradients made in the corresponding buffer. The radioactivity profiles obtained are shown in Fig. 3.9. No significant interaction was found when viral RNA was allowed to interact with purified 14S particles in Tris buffer (panel A). However, aggregates were observed around the 100S region when sodium phosphate was used as a dialysis buffer (panel B), and the amounts of these aggregates increased with the amount of RNA in the incubation mixture. Similar results were obtained when purified 14S particles from infected cells were used.

Because of the effect of detergent on the recovery of purified 14S particles, the 14S particle-RNA mixtures and control samples (14S particles alone) were dialyzed for 5 hr against sodium phosphate in the presence of 1% NP40, and were analyzed by centrifugation in a 15-30% sucrose gradient made in sodium phosphate and containing 1% NP40. A defined peak sedimenting at the position of intact Mengo virions was obtained with the 14S particles from infected cells (Fig. 3.9, panel C) but not with those from the reticulocyte lysate (data not shown). These results suggest that there is an interaction between Mengo RNA and the 14S particles and

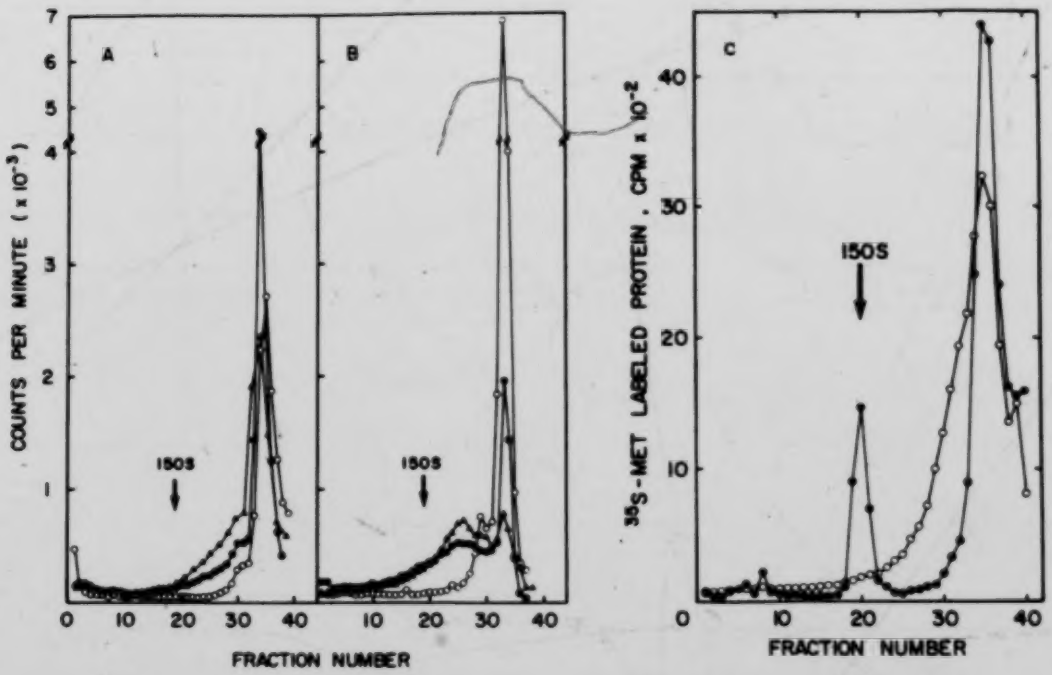

Fig. 3.9 Interaction of purified Mengo 14S particles with virion RNA: sucrose gradient sedimentation analysis.
Mengo 14S particles from the *in vitro* translation system (panels A and B) or from infected L-cells (panel C) were displaced from the immunoaffinity column by 13.4S capsid subunits as described in the text. One ml of the column effluent (in RSB) was mixed with 25 µg Mengo virion RNA (Δ-Δ), 7.6 µg Mengo virion RNA (•-•) or no RNA (o-o), and the mixtures dialyzed against either 0.1 M KCl, 0.01 M Tris, pH 7.3 (panel A) or 0.1 M sodium phosphate, pH 7.2 (panel B), or 0.1 M sodium phosphate, pH 7.2, 1% NP40 (panel C). Centrifugation was in 15-30% sucrose gradients made with the corresponding buffer. [^{3}H]leucine labelled Mengo virions (150S) were centrifuged in parallel gradients to provide a sedimentation marker.

that this interaction may be different for 14S particles obtained from *in vivo* and *in vitro* sources. However, the morphology, the protein composition and the presence of RNA in the 150S structure have yet to be determined.

Since the purified 14S particle preparation contained a preponderance of 13.4S capsid subunits, it was necessary to determine whether the subunits themselves could form aggregates with the viral RNA. Were this type of interaction to occur, it would be possible that the radiolabelled 14S particles could simply have been trapped in such aggregates. Therefore, incubation of [^{3}H]leucine labelled 13.4S capsid subunits with RNA was carried out under the same conditions. In these experiments no radiolabelled material was found sedimenting in structures larger than 13.4S (data not shown). Thus, the 13.4S subunits neither formed complexes with the viral RNA nor interfered with the capacity of the 14S particles to do so.

The monomeric 5S particles eluted from the immunoaffinity column by LIS were also incubated with viral RNA and the mixtures dialyzed against 0.1 M sodium phosphate (pH 7.2). In this instance, no significant amounts of radioactivity were found to sediment outside the 5S region of the gradient. There was an indication of a faster-sedimenting shoulder to the 5S peak, and a small pellet was recovered from the bottom of the tube following centrifugation. However, an identical distribution of radioactivity was found in a control experiment in which the

viral RNA was omitted from the incubation mixture. Composition analysis by SDS-PAGE and autoradiography showed that the 5S peak, the faster sedimenting shoulder and the pellet all contained roughly equimolar amounts of ε, α and γ, and traces of D1, G and V. Thus, the LIS-eluted 5S particles were incapable of interaction with Mengo RNA under the incubation conditions used.

3.5 Examination of the Amino Terminus of Precursor Protein ε

3.5.1 Isolation and Attempts to Determine the N-terminal Sequence of ε

Ziola and Scraba (1974) showed that the δ polypeptides in purified Mengo virions have no free N-terminal amino groups. This is also true of the smallest capsid protein of other picornaviruses (for a review, see Putnak and Phillips, 1981). These observations raised questions as to whether the blocking reaction accompanies final virion assembly or instead occurrs at the polypeptide precursor stage, and regarding the nature of the blocking group. Dorner *et al.* (1982) have provided convincing, if negative, data to support the idea that blocking occurs at the polypeptide precursor stage for poliovirus: automated Edman sequencing failed to release any free amino acids from δ, ε or B, even though digestion with trypsin and subsequent automated sequencing (of the tryptic peptides) showed that these were the authentic viral proteins. Similar results have been

obtained with the precursor proteins of FMD virus (Robertson *et al.*, 1985). It was therefore interesting to attempt to ascertain whether the amino terminal of ε component of Mengo virus 14S particles is also blocked.

The simplest procedure for answering this question is to isolate and sequence the amino terminal of ε from the immunoaffinity purified 14S or 5S particles. Since a substantial amount of dissociated virus was required to displace intact 14S particles from the column, and the effluent would contain a preponderance of 13.4S capsid subunits, the LIS eluted 5S particles (dissociated 14S particles) were used as the source of ε proteins. Individual viral proteins can be separatd from the protomer structure and other possible contaminating proteins by SDS-PAGE and recovered by electrophoretic elution from the gel (see Materials and Methods). The resulting pure ε protein could then be subjected to either automated or manual (Chang *et al.*, 1978) amino terminal sequencing.

Conditions for electroelution and sequencing were examined using purified Mengo virions before the actual isolation and sequencing of ε was performed. Capsid proteins α, β, γ and δ from 100 μg of [^{3}H]leucine labelled Mengo virus (~1 nanomole, ~1x10^8 cpm) were separated by electrophoresis in phosphate-urea or Laemmli gels and were electroeluted as described in the Materials and Methods. A segment of gel containing no detectable radioactive protein was excised and used as a control sample to obtain a

background for the manual sequencing. The total recovery of proteins was estimated to be about 50% from Laemmli gels, but only about 5% from phosphate urea gels.

Portions of the electroeluted proteins were analyzed by standard phosphate-urea SDS-PAGE and autoradiography. Neither degradation of the proteins nor contamination by other radioactive proteins was detected. The eluted proteins were then subjected to manual N-terminal sequencing as described by Chang *et al.* (1978), and 2 nanomoles of myoglobin was sequenced in parallel. The small amounts of SDS (0.02% after electroelution) in the capsid protein samples altered the migration of modified amino acids on the TLC plates, making identification impossible. Electroeluted proteins were therefore dialyzed extensively against pyridine (which was the solvent for the protein during manual sequencing) in order to remove the SDS, and at the same time to prevent precipitation of the protein. Recovery of radioactivity after dialysis was about 30%. Thus, although the SDS was removed and the proteins could be sequenced, the amounts of protein recovered in the dialysates were so low that only the first two sequencing cycles could be performed. The N-terminal amino acids of β were identified as Asp-Gln and the second amino acid from the N-terminus of γ was shown to be proline. These results confirmed the amino-terminal sequences of these proteins determined by Ziola and Scraba (1976). The first sequencing cycle of γ, however, did not reveal any modified amino acid.

As reported by Ziola and Scraba (1976), the first N-terminal amino acid of γ is serine. Recovery of modified serine by this manual sequencing method was found to be too low for satisfactory identification if the amount of the starting material was also low (Chang *et al.*, 1978). Therefore, it was not surprising that the N-terminal serine of γ was not detected.

To collect enough material for the sequencing of ε, 5S particles eluted by LIS from several immunoaffinity columns were dialyzed against dilute ammonia to remove the LIS. Only small losses accompanied this step. The dialysates were then freeze-dried from water three times to remove the ammonia. The resulting insoluble yellow powder was suspended in 50 μl volumes of glass distilled water and aliquots of the suspensions were analyzed by standard SDS-PAGE and autoradiography. The proteins in about half of the samples were found to have been degraded during the preparation and were useless for sequencing. For the samples still containing intact proteins, the recovery of [^{35}S] radioactivity was only about 25% of the amount in the original column eluant. It was calculated that in order to gather enough material for the electroelution and manual sequencing, about 150 ml of infected L-cell lysates (*i.e.* 30 roller bottles of infected cells and 150 sucrose gradients) would have to be prepared. Since this was neither practical nor economical, we decided to sequence the 5S particles (containing equimolar amounts of ε, α and γ) directly

without isolating the individual proteins and encountering further losses during electroelution and removal of the SDS. Theoretically, only two amino-termini should be revealed if the N-terminus of ϵ were blocked, and the amino acid derivatives generated from α, γ should be present in approximately equimolar amounts. Since some impurities had been shown by silver staining to be present in the 5S particle-containing fractions eluted from the immunoaffinity column (see section 3.3.2), a quantitative estimation of the amino acid derivatives was necessary to distinguish impurities from authentic ϵ, α and γ proteins. While it would be very difficult to quantitate the amounts of amino acid derivatives separated on TLC plates by the manual method, automated sequencing would permit quantitative estimation of the amino acids by HPLC, and was thus the method of choice. Consequently 0.64 nanomoles of freeze-dried 5S material was collected for sequence determination in an automated gas-phase sequenator. The dried sample was not soluble in trifluoroacetic acid but was partially dissolved in 100 μl 25% NH_4OH by vortex mixing and sonication. The solution obtained was yellow in colour, and a white powder was left undissolved. The sequencing was begun following application of the yellow solution onto a glass-fibre filter disk and drying. However, no detectable amino acids were released in ten sequencing cycles. It is possible that the protein was, in fact, the white powder which had not dissolved, and that only a yellow coloured

impurity was extracted and subjected to sequence determination. These efforts to determine whether or not the Mengo ε polypeptides have a free N-terminal were, therefore, not successful.

3.5.2 Attempts to Detect Acetylated Mengo Capsid Presursor Protein(s) Synthesized *In Vitro*

The most common amino-terminal blocking group found in proteins produced by eukaryotic cells and their viruses is the acetyl group (Tsunasawa and Sakiyama, 1984; Wold, 1984). The possibility of the capsid precursors A, B and/or ε being acetylated proteins was therefore examined using the *in vitro* synthesis system. [^{3}H]-acetyl CoA was added to the Mengo RNA programmed reticulocyte lysate during a 5 hr incubation period in order to label acetylated proteins. To avoid overloading the SDS-PAGE analytical gel with reticulocyte proteins, Mengo capsid protein products were first immunoprecipitated with the anti-β antibody-conjugated Sepharose beads used for the affinity column. After washing twice with RSB, the Mengo proteins were extracted from the precipitate with the electrophoresis 2x sample buffer (see Materials and Methods). When the gels were subjected to autoradiography following electrophoresis, all three structural proteins (ε, α and γ) were found to be labelled. This was unexpected because the amino termini of α and γ are known to be unblocked (Ziola and Scraba, 1976), and should not have been acetylated. This result suggested that, in

spite of the presence of five times the normal concentration of nonradioactive amino acids (100 μM total amino acids) in the incubation mixture, [^{3}H]-acetyl CoA had been metabolized and incorporated into amino acids which were then inserted into the newly synthesized viral proteins (Palmiter, 1977).

The experiment was therefore repeated, this time with the addition of unlabelled acetyl CoA to a final concentration of 2 mM after an initial 20 min incubation time. The assumption was that the radioactive acetyl CoA would be incorporated into the amino terminals of the newly synthesized capsid protein precursors or their cleavage products during the first 20 min of protein synthesis, and that the addition of unlabelled acetyl CoA would dilute the pool of unincorporated radioactive acetyl CoA. Thus the metabolic conversion to amino acids would result only in low levels of general incorporation of radioactivity into viral proteins formed during the remainder of the 5 hr incubation. Equal volumes of incubation mixtures with or without added Mengo RNA were withdrawn just before the addition of unlabelled acetyl CoA, and after 5 hr. The capsid proteins in incubation mixtures containing RNA were collected by immunoprecipitation. As controls, the incubation mixtures without RNA were treated with Sepharose beads coupled to anti-bovine serum albumin antibodies. The samples were analyzed by SDS-PAGE and autoradiography. All three viral structural proteins were again found to be labelled in the 5 hr sample from the RNA programmed lysate (3 weeks exposure

of the gel to the X-ray film). However, no labelled protein band was detected in the fluorogram of the 20 min sample, even after three months exposure. It was concluded that the [^{3}H]-acetyl CoA molecules were again converted into amino acids during the 5 hr incubation period, and that it is not possible to detect N-terminal acetylated capsid precursor proteins (if such were indeed present) by this method.

3.5.3 Effects of Oxalacetate and Citrate Synthase on the Cell-free Synthesis and Processing of Mengo Virus Proteins

The possibility that polypeptide ε synthesized *in vitro* is acetylated at its amino terminal was also examined by depleting the reticulocyte lysate of acetyl CoA by the action of citrate synthase (CS) in the presence of oxaloacetate (OAA) (Palmiter, 1977; Paucha *et al.*, 1978; Anderson and Lewis, 1980). Proteins synthesized in this modified system would not be expected to be acetylated, and should therefore be amenable to sequencing by automated procedures. They might also have altered mobilities during SDS-PAGE. However, the synthesis of all Mengo viral proteins was found to be drastically inhibited when CS (30 Units/ml) and OAA (1 mM) were added to the *in vitro* protein synthesizing system. These results are shown in Fig. 3.10, lane 1. Incorporation of [^{35}S]methionine was only 5-20% of normal and, remarkably, only the structural protein precursors A1, A, B and occasionally traces of D1 and

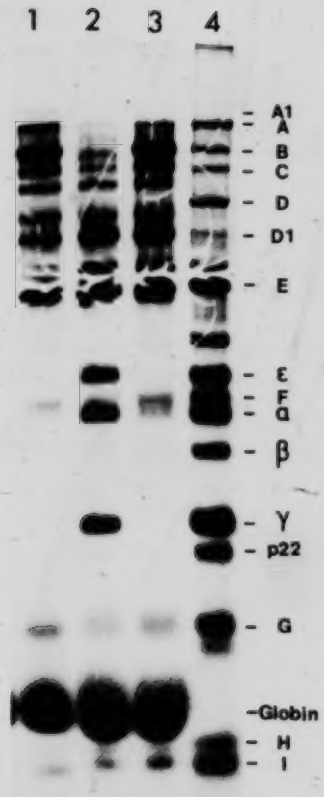

Fig. 3.10 Processing of the Mengo virus polypeptides in the rabbit reticulocyte system in the presence of oxaloacetate (OAA) and citrate synthase (CS): SDS-PAGE analysis.
A rabbit reticulocyte lysate containing [^{35}S]methionine was supplemented with 1 mM OAA and 30 units CS/ml (final concentrations). Mengo RNA was added and the mixture incubated for 5 hr before a sample was removed for SDS-PAGE analysis. The polypeptide pattern of this sample is shown in lane 1 of the autoradiogram. Lane 2 shows the pattern when an unlabelled, Mengo RNA programmed reticulocyte lysate (no OAA or CS) was added to the sample of lane 1 and incubation continued for an additional 5 hr prior to electrophoresis. Lane 3 shows the pattern from a sample prepared in an identical manner to that of lane 2 except that the added (unlabelled) reticulocyte lysate had not been programmed for protein synthesis by Mengo RNA. Lane 4 shows the profile of a reference lysate from Mengo virus infected L-cells at 6 hr post infection; [^{35}S]methionine was the label. The procedures for electrophoresis and autoradiography are described in Materials and Methods.

nonstructural proteins were visible in the autoradiogram, even after 16 hr incubation. This observation suggested that OAA and CS had somehow impaired both protein synthesis and proteolytic processing. Accordingly, to the system containing OAA and CS, was added an equal volume of a normal Mengo RNA programmed 5 hr incubation mixture (no radioactive amino acids). The incubation mixture added presumably contained the viral protease (p22), since processing of A1, A and B into D1, ϵ, α and γ was observed (Fig. 3.10, lane 2). No such processing was observed with the addition of an unprogrammed lysate (lane 3). It was concluded that the viral protease was either non-functional or synthesized in only minute amounts in the reticulocyte system in the presence of OAA and CS. Different amounts of OAA or CS were then added separately to the cell-free system immediately before the 5 hr incubation. As shown in Fig. 3.11, the inhibition of viral protein synthesis and processing was directly related to the concentrations of either or both 'inhibitors'. When relatively small amounts of the 'inhibitors' were present, viral precursor proteins were synthesized and processed, but the cleavages of D1 $\rightarrow \epsilon + \gamma$ and sometimes B $\rightarrow$ D1 + α were much less efficient than normal. Similar observations were made when incubation mixtures were supplemented with 1 mM OAA and 30 U CS/ml after 20 min incubation. These observations suggest that in the *in vitro* translation system, OAA and CS inhibit both the initiation of Mengo RNA directed protein synthesis and the

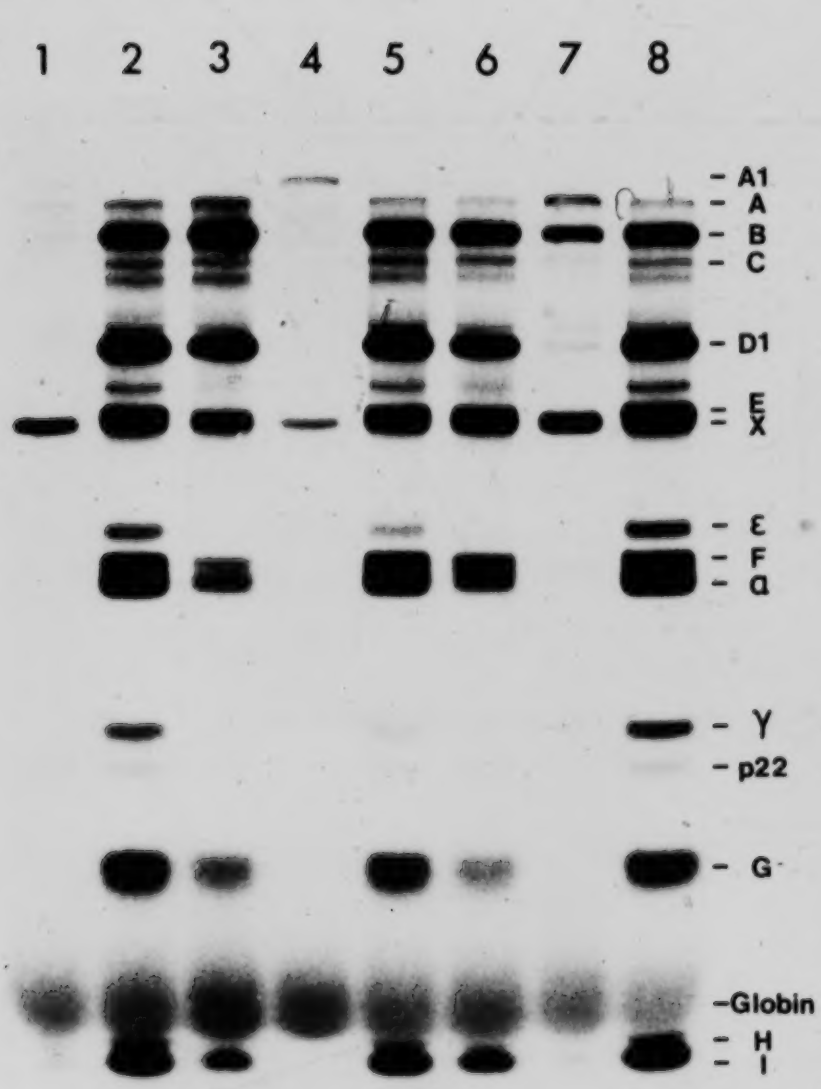

Fig. 3.11 Effects of different concentrations of oxaloacetate (OAA) and citrate synthase (CS) on the synthesis of Mengo virus proteins in the rabbit reticulocyte translation system: SDS-PAGE analysis.
Prior to SDS-PAGE and autoradiography, rabbit reticulocyte lysates containing [^{35}S]methionine and programmed with Mengo RNA were supplemented with the following final concentrations of OAA and CS: 1 mM OAA and 30 U CS/ml (lane 1); 0.2 mM OAA (lane 2); 1 mM OAA (lane 3); 2 mM OAA (lane 4); 5 U CS/ml (lane 5); 10 U CS/ml (lane 6); 30 U CS/ml (lane 7); and no OAA or CS (lane 8). Incubation in all cases was for 5 hr at 30°.

enzymatic activity of the viral protease.

However, eukaryotic and viral proteins with unblocked N-termini have been synthesized under the direction of capped messenger RNA's in *in vitro* systems containing OAA and CS, and the amount of inhibition was reported to be less than 25% (Palmiter, 1977; Paucha *et al.*, 1978). It seemed strange that translation of Mengo RNA was markedly inhibited even when CS alone was added. To further investigate the action of OAA and CS in the reticulocyte lysate system, RNA's from Cowpea mosaic virus (CpMV) and Brome mosaic virus (BMV) were translated in reticulocyte lysate in the presence of these 'inhibitors'. CpMV genomic RNA's are, like Mengo RNA, uncapped messengers with VPg proteins covalently attached to their 5'-termini. Also, amino acid sequence homologies with picornaviruses have been found in the C-terminal region of the protease (MW 24,000) and the entire polymerase region of CpMV (Argos *et al.*, 1984; Franssen *et al.*, 1984). These two viruses also have similar gene arrangements, with capsid proteins being cleaved from a large precursor, and the viral protease and polymerase from another precursor (see Fig. 3.12). However, the structural and nonstructural proteins of CpMV are encoded in two different RNA molecules (the M and B RNA's, respectively). As shown in Fig. 3.12, the translation of CpMV M RNA alone gives rise to a capsid precursor of MW 105,000 or 95,000, which will not be processed unless the protease encoded in the B RNA is present (Pelham, 1979; Franssen *et al.*, 1982).

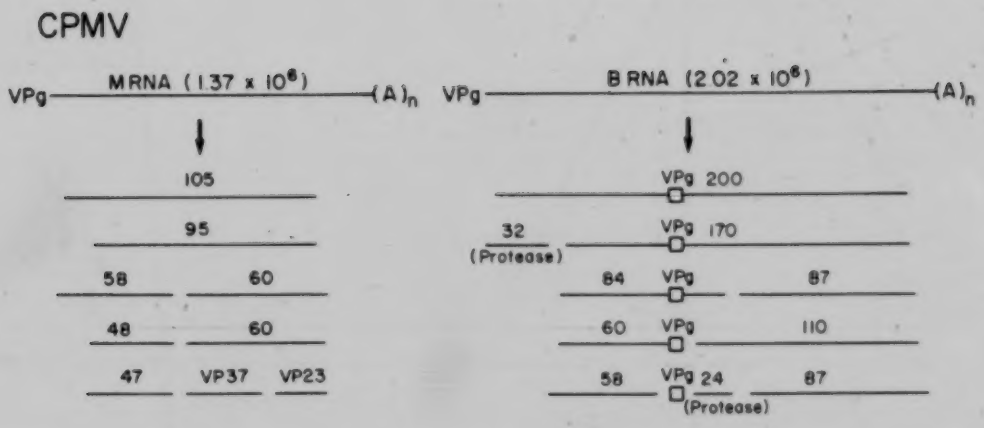

Fig. 3.12 Model for the synthesis and post-translational processing of Cowpea mosaic virus (CpMV) proteins (from Goldbach and Rezelman, 1983).
Both the middle component RNA (M RNA) and the bottom component RNA (B RNA) of CpMV are 5' VPg-linked and 3' polyadenylated. B RNA encodes a polyprotein of MW 200,000 which is processed by proteolysis to yield a protease (MW 32,000) and a smaller polyprotein (MW 170,000). Autoproteolysis of the latter by an intrinsic protease (MW 24,000) is thought to produce the final 4 nonstructural polypeptides. The M RNA encodes the two capsid polypeptides, VP37 and VP23. It is believed that the 32,000 MW protease causes the cleavages of the M polyproteins (MW 105,000 and 95,000) to precursors of MW 58,000, 48,000 and 60,000. The latter precursor is cleaved to produce the capsid proteins by an enzyme present in the leaves of infected plants, but not in an *in vitro* translation system (Franssen *et al.*, 1982).

Translation of the B RNA alone gives rise to this viral protease (of MW 32,000) and a postulated protease (MW 24,000), which is thought to generate itself and the other nonstructural proteins from the precursor (MW 170,000) by an autocatalytic process. Because of the bipartite nature of the CpMV genome, one could theoretically examine separately the action of OAA and CS on the initiation of protein synthesis and on the enzymatic activity of the viral protease (by the translation of either M or B RNA *in vitro* in the presence of these 'inhibitors'). BMV mRNA's, on the other hand, are capped (with m'G(5')ppp(5')N^{m}--), and four separate proteins are encoded in four separate mRNA's. Strong inhibition of protein synthesis *in vitro* by OAA and CS would not be expected if the BMV messenger RNA molecules behave the same as those other capped cellular and viral mRNA's which have been studied.

It was hoped that the CpMV M and B nucleoprotein components which had been separated by one equilibrium centrifugation in cesium chloride would be pure enough to isolate M and B RNA without cross-contamination. However, the *in vitro* translation products of the M RNA preparation were contaminated by products of B RNA, including the protease, and processing of the M RNA-coded capsid precursor occurred. It was estimated from the relative amounts of the various proteins synthesized in the translation mixture, that roughly 15% of the total M RNA preparation was B RNA. Therefore, it was necessary to examine the action of OAA and

CS on M and B RNA's together or on B RNA alone. If the protease were inhibited, no processing of the M RNA products would be expected; whereas if the initiation of protein synthesis were inhibited, then both the M RNA and B RNA's would be translated to only a limited extent. As shown in Fig. 3.13, translation of all of the viral RNA molecules was inhibited. The extent of inhibition was 39%, 61-64% and 84% for BMV RNA's, CpMV RNA's and Mengo RNA, respectively (see Table 3.3). The data indicate that OAA and CS are able to inhibit the initiation of translation of uncapped messengers more efficiently than they inhibit translation of capped messengers, and that among the uncapped messengers examined here, the translation of Mengo RNA is most effectively inhibited. It appears from the autoradiogram shown on Fig. 3.13 (lane 1 and 3) that the translation of both CpMV RNA's in the presence of OAA and CS have been abolished. However, upon longer exposure time, precursor protein bands and some of their cleavage products became visible. Thus the 'inhibitors' did not seem to reduce the activities of the proteases of CpMV.

The particular susceptibility of Mengo RNA to inhibition by OAA and CS might suggest the existence of unique structural features near its 5'-end or it may be that the poly(C) tract which precedes the coding region plays a role in the inhibition. Nevertheless, it is clear that because the initiation of protein synthesis by Mengo RNA is severely impaired, it is not possible to use OAA and CS at

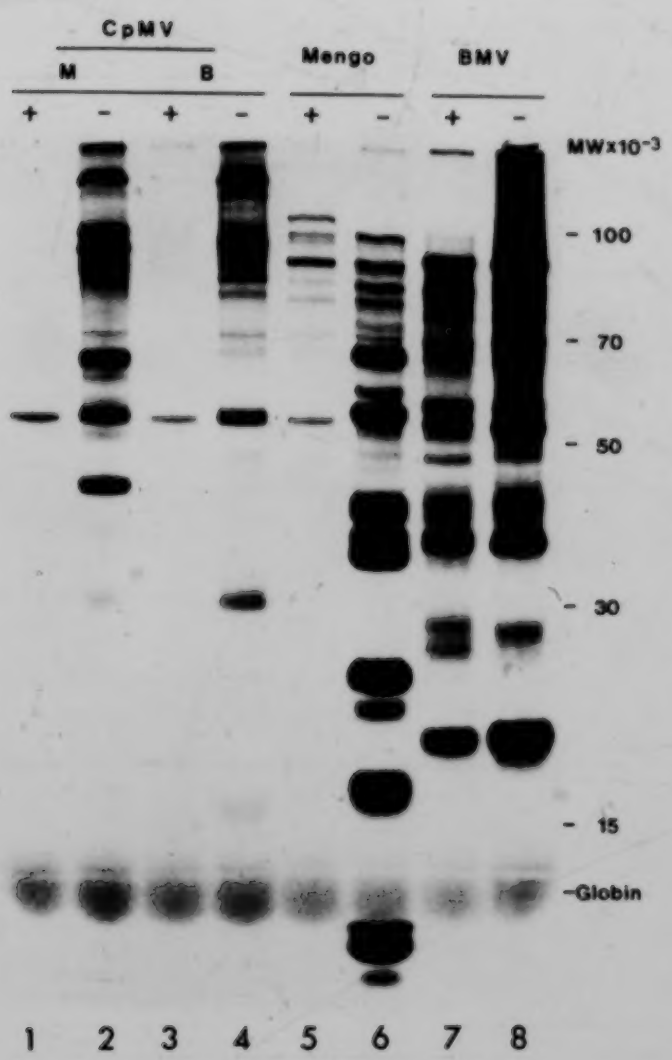

Fig. 3.13 Effect of oxaloacetate (OAA) and citrate synthase (CS) on the *in vitro* translation of viral RNA's: SDS-PAGE analysis.
The translation of Cowpea mosaic virus M RNA (with 15% contamination of B RNA; lanes 1 and 2), CpMV B RNA (lanes 3 and 4), Mengo RNA (lanes 5 and 6) and Brome mosaic virus (BMV) RNA (lanes 7 and 8) were carried out in the rabbit reticulocyte system in the presence of [^{35}S]methionine for 5 hr at 30°. OAA and CS were included in the lysates for lanes 1, 3, 5 and 7 (designated as '+') at final concentrations of 1 mM and 30 U/ml, respectively. The conditions for electrophoresis and autoradiography are described in the Materials and Methods. The molecular weight values given on the right were approximated from the migration rates of the Mengo proteins shown in lane 6.

Table 3.3 Inhibition of translation by 1 mM oxaloacetate and 30 U citrate synthase/ml.

Viral RNA used as messenger	5' Structure	Total protein synthesized (% of control)'
CpMV M and B RNA	VPg-pN--	36%
CpMV B RNA	VPg-pN--	39%
Mengo RNA	VPg-pN--	16%
BMV RNA	m'G(5')ppp(5')N^{m}--	61%

'Control: Total protein synthesized in the absence of OAA and CS.

these concentrations in order to synthesize unacetylated Mengo capsid proteins *in vitro*.

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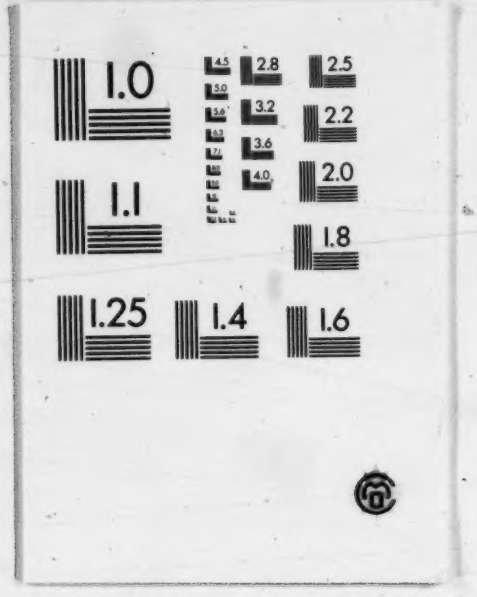

4. DISCUSSION

It has been proposed that a 14S particle ($[\epsilon\alpha\gamma]_5$) is one of the major structural intermediates for picornavirus assembly (Watanabe *et al.*, 1962; Phillips *et al.*, 1968; also see Fig 1.3). This particle was found in Mengo virus infected L-cells and has been isolated from Mengo RNA programmed rabbit reticulocyte lysates. Sucrose density gradient centrifugation and SDS-PAGE analysis have revealed that the polypeptide composition and sedimention behaviour of 14S particles from the two sources are similar, if not identical.

In the cell-free translation system, synthesis of the capsid protein precursor A1 was detected. Cleavage of this precursor follows the A1 → A → B → D1+α → ϵ+α+γ route, and the resultant capsid proteins remain associated as a 5S protomer [$\epsilon\alpha\gamma$]. Although it has been proposed that other forms of 5S protomer (*e.g.* A) and 13S pentamer (*e.g.* $[A]_5$) may be precursors of the 14S particle in EMC and rhinovirus infected cells (McGregor *et al.*, 1975; McGregor and Rueckert, 1977), the results presented here show clearly that in the reticulocyte lysate, the cleavage of precursors in the 5S protomers precedes the formation of the 14S pentamer ($[\epsilon\alpha\gamma]_5$) (Fig. 3.3 and Fig. 3.4). Assembly of Mengo viral structural precursors in the cell-free system is limited to the 5S and 14S particles; no larger viral structures were found, even after prolonged incubation. Moreover, the final cleavage of ϵ to β and δ, which

accompanies the encapsidation of viral RNA, does not occur in the cell-free translation system.

14S particles isolated from poliovirus infected cells were found to assemble into 80S empty shells, similar to those produced in infected cells, in the presence of infected cell lysates (Phillips *et al.*, 1968). These *in vitro* assembly studies suggested that the formation of empty capsids occurrs before the viral RNA interacts with the capsid proteins. In order to carry out similar studies of Mengo virus assembly, the 14S peak material obtained from sucrose gradients was first subjected to a 'homochromatographic' purification procedure. This involved the displacement of 14S particles from an immunoaffinity column containing monoclonal antibodies which recognized the structural protein β by 13.4S Mengo capsid subunits. This procedure removed most of the contaminating cellular and nonstructural viral proteins from the 14S particle without disrupting its integrity (Fig. 3.6 and 3.7). Preliminary assembly studies *in vitro* showed that, although these purified 14S particles do not assemble into 80S empty capsids by themselves, they do interact with viral RNA to form structures which sedimented at 100S or more. This interaction was not due to the presence of 13.4S capsid subunits in the 14S particle preparation; no structure larger than 13-14S was found when radioactive 13.4S capsid subunits were allowed to interact with Mengo RNA.

We have now obtained (from Mr. J. Bowen in Dr. J.S. Colter's laboratory) a monoclonal antibody which is specific for dissociated virions (*i.e.* 13.4S particles) but does not bind to the 14S particles. Experiments are currently underway to see whether this antibody can be used to remove the 13.4S subunits from the 14S preparations. Further investigations will involve the testing of various conditions to obtain consistent results from the RNA-14S particle assembly reactions. Also, [^{3}H]uridine labelled Mengo viral RNA will be used in order to elucidate the stoichiometry of RNA in the structures produced *in vitro*.

While 14S particles obtained from both Mengo virus infected cells and rabbit reticulocyte lysates were indistinguishable from one another in terms of polypeptide composition and sedimentation behaviour, they did exhibit some differences. The formation of 14S particles in the cell-free system is a relatively slow process which takes almost 16 hr to complete as opposed to the 4 hr period required for 14S particle formation in infected L-cells. This seems to be more than a concentration effect, and suggests the involvement of a morphopoietic factor in L-cells which is not present (or not active) in the rabbit reticulocyte lysate. Also, a higher proportion of 14S particles from infected cells than 14S particles from the cell-free system can be displaced from the immunoaffinity column by the 13.4S subviral particles. This implies that the organization of the proteins in 14S particles formed

in vivo and *in vitro* differed in a measurable way. Preliminary experiments showed that interaction of the 14S particles with Mengo RNA was also different for particles obtained from *in vitro* and *in vivo* sources. These observations, taken together with the absence of the $\epsilon \rightarrow \beta + \delta$ cleavage in the reticulocyte translation system, suggest that the one or two copies of immature pentamers normally found in each Mengo virion might follow a similar morphogenetic pathway to that of the 14S particles produced *in vitro*. A morphopoietic factor present in L-cells might be required to convert these 14S particles into particles which can be cleaved (autoproteolysis of ϵ?) and assembled into mature virions. Therefore, an examination of the effects of L-cell lysates (either infected or uninfected) or of fractions produced therefrom on the interaction of 14S particles from both sources should provide further insights into the assembly process.

The final step of picornavirus maturation involves the cleavage of ϵ to the capsid proteins β and δ. Since the amino-terminus of δ is blocked (Ziola and Scraba, 1976), it might be postulated that the reduction of positive charges brought about by this N-terminal modification occurs to facilitate virion assembly. Alternatively, the N-terminal modification might occur at an early stage of post-translational processing, in order to protect the original amino terminal of the polyprotein from exopeptidases. To decide between these two alternatives, an attempt was made

to ascertain whether or not the Mengo ε polypeptide has a free amino terminal. 14S particles from infected cells were concentrated by sucrose gradient centrifugation, specifically bound to the immunoaffinity column, and eluted with LIS. The resulting 5S particles were dialyzed and freeze-dried, and this equimolar mixture of ε, α and γ was subjected to automated N-terminal sequencing. Only two amino-termini should have been revealed if ε lacks a free amino-terminus. Unfortunately, the first attempt at sequencing was unsuccessful, mainly due to the insolubility of the sample after freeze-drying. Therefore, freeze-drying is not recommended for future trials. The concentration of the 5S materials in the LIS eluant was too low for direct automated sequencing in the first trial. In order to increase the concentration, more 14S materials could be applied to the immunoaffinity column, and the dialyzed eluant could be subjected to vacuum evaporation without being dried completely. Dilute ammonia containing small amounts of SDS (0.02% SDS should not interfere with the automatic sequencing; Hunkapiller *et al.*, 1983) could then be used to dissolve the sample for sequence studies. If the presence of α and γ or other contaminating proteins were to present a problem for analysis of the automated sequencing results, the concentrated 5S sample could be subjected to electrophoresis in Laemmli gels and the individual proteins electroeluted before sequencing. Since the automated sequenator is able to sequence the amino-termini of

picomoles of proteins (Hewick *et al.*, 1981), sequence determinations of the first few amino acid residues of each of the α and γ proteins, and of ε if it is not blocked, should be possible dispite losses occurring during electroelution.

Efforts to produce non-acetylated ε proteins in a modified cell-free translation system were also made. Failure to label any viral protein with [^{3}H]-acetyl CoA during a 20 min incubation time might imply that the precursor protein A1 was not acetylated. However, the antibody employed to immunoprecipitate the capsid proteins prior to SDS-PAGE analysis was specific for the 13.4S viral particles, and soluble proteins like A1 and A may not have been efficiently removed from solution. On the other hand, the conversion of [^{3}H]-acetyl CoA into amino acids obviated any meaningful interpretation of samples incubated for 5 hr. Addition of more unlabelled amino acids (for example, 100 μM of each) is therefore recommended for future experiments. Immunoprecipitation of hemoglobin from the incubation mixture followed by TCA precipitation of the proteins, instead of preferentially immunoprecipitating the proteins in the 5S or 14S particles, may be a better procedure for detecting acetylated precursor proteins at early incubation times.

The failure of microsequencing to reveal an amino terminal amino acid in precursor B and ε synthesized in the rabbit reticulocyte lysate, and of B synthesized in the

acetylation-inhibited lysate programmed by FMD virus RNA has recently been reported (Robertson *et al.*, 1985). Since normally acetylated proteins would be synthesized in the acetylation-inhibited lysate in an unblocked form, failure to sequence precursor B produced in such a system suggested that the N-terminal of B (and probably ϵ and δ) might be blocked by something other than an acetyl group. Since protein synthesis directed by Mengo RNA is very effectively inhibited in the cell-free system supplemented with OAA and CS, it was somewhat surprising to find that amounts of proteins adequate for automated sequencing could be synthesized in a similar system when FMD virus RNA was used as messenger. Dorner *et al.* (1982) had failed to synthesize unblocked precursor A in similar acetylation-inhibited cell-free system programmed by poliovirus RNA. The reason for these differences between the Mengo and poliovirus RNA directed translation systems on the one hand and the FMD RNA directed system on the other, are not clear.

Although nonacetylated Mengo proteins (*e.g.* ϵ) were not synthesized in the acetylation-inhibited, cell-free system, the effects of OAA and CS on the *in vitro* translation of Mengo RNA are perhaps worthy of further study. The inhibitory effect of 1 mM OAA and 30 U CS/ml on the translation of Mengo RNA found in our experiments was not observed with ovalbumin mRNA (Palmiter, 1977), and only partial inhibition occurred with BMV RNA (these studies) and SV40 large tumor antigen mRNA (Paucha *et al.*, 1978). These

results imply that differences may exist among mRNA molecules with respect to the mechanisms of initiation of protein synthesis. Processing of D1 by the Mengo viral protease was also affected by either or both OAA and CS; the mechanisms of these inhibitions are not clear. More detailed studies of these compounds may prove to be useful for understanding both the initiation of protein synthesis and the proteolytic processing of the picornaviral proteins.

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